PCT WORLD INTELLECTUAL PROP
International B

INTERNATIONAL APPLICATION PUBLISHED UNDEI

(51) International Patent Classification 6 :
A61K 39/12, 39/21, C07K 14/16, C12N 15/34

A2

(11) I

WO 9609066A2

(43) International Publication Date: 28 March 1996 (28.03.96)

(21) International Application Number: PCT/US95/11943

(22) International Filing Date: 19 September 1995 (19.09.95)

(30) Priority Data:
08/311,424 23 September 1994 (23.09.94) US

(71) Applicants: THE JOHNS HOPKINS UNIVERSITY SCHOOL OF MEDICINE [US/US]; 720 Rutland Avenue, Baltimore, MD 21205 (US). THE ADMINISTRATORS OF THE TULANE EDUCATIONAL FUND [US/US]; 1430 Tulane Avenue SL76, New Orleans, LA 70112-2699 (US).

(72) Inventors: CLEMENTS, Janice, E.; 10965 Shadon Lane, Columbia, MD 21044 (US). MURPHEY-CORB, Michael; 449 South America Street, Covington, LA 70433 (US). ZINK, M., Christine; 1907 Eastridge Road, Timorium, MD 21093 (US).

(74) Agent: HAILE, Lisa, A.; Fish & Richardson P.C., Suite 1400, 4225 Executive Square, La Jolla, CA 92037 (US).

(81) Designated States: AM, AT, AU, BB, BG, BR, BY, CA, CH, CN, CZ, DE, DK, EE, ES, FI, GB, GE, HU, IS, JP, KE, KG, KP, KR, KZ, LK, LR, LT, LU, LV, MD, MG, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, TJ, TM, TT, UA, UG, UZ, VN, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG), ARIPO patent (KE, MW, SD, SZ, UG).

Published
Without international search report and to be republished upon receipt of that report.

(54) Title: METHOD OF TREATMENT OF HUMAN IMMUNODEFICIENCY VIRUS (HIV) INFECTION

(57) Abstract

A method of treating a host prophylactically or therapeutically for infection with human immunodeficiency virus (HIV) is described. A simian immunodeficiency virus (SIV)-macaque monkey model shows that infection with a non-pathogenic, macrophage-tropic, recombinant SIV provides systemic immunity to the host by stimulation of production of neutralizing antibodies. Tests provided show that the neutralizing antibodies produced are broadly reactive with several heterologous strains of SIV. Also provided are pharmaceutical compositions useful for treatment of a subject prior to or following infection with HIV.

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AT	Austria	GB	United Kingdom	MR	Mauritania
AU	Australia	GE	Georgia	MW	Malawi
BB	Barbados	GN	Guinea	NE	Niger
BE	Belgium	GR	Greece	NL	Netherlands
BF	Burkina Faso	HU	Hungary	NO	Norway
BG	Bulgaria	IE	Ireland	NZ	New Zealand
BJ	Benin	IT	Italy	PL	Poland
BR	Brazil	JP	Japan	PT	Portugal
BY	Belarus	KE	Kenya	RO	Romania
CA	Canada	KG	Kyrgystan	RU	Russian Federation
CF	Central African Republic	KP	Democratic People's Republic of Korea	SD	Sudan
CG	Congo			SE	Sweden
CH	Switzerland	KR	Republic of Korea	SI	Slovenia
CI	Côte d'Ivoire	KZ	Kazakhstan	SK	Slovakia
CM	Cameroon	LI	Liechtenstein	SN	Senegal
CN	China	LK	Sri Lanka	TD	Chad
CS	Czechoslovakia	LU	Luxembourg	TG	Togo
CZ	Czech Republic	LV	Latvia	TJ	Tajikistan
DE	Germany	MC	Monaco	TT	Trinidad and Tobago
DK	Denmark	MD	Republic of Moldova	UA	Ukraine
ES	Spain	MG	Madagascar	US	United States of America
FI	Finland	ML	Mali	UZ	Uzbekistan
FR	France	MN	Mongolia	VN	Viet Nam
GA	Gabon				

METHOD OF TREATMENT OF HUMAN IMMUNODEFICIENCY VIRUS (HIV) INFECTION

This invention was made with Government support by Grant No. PO1 NS32208 from the National Institutes of Health, NINDS, and Grant No. UOIAI28243 from the National Institutes of Health, NIAID. The Government has certain rights in the invention.

BACKGROUND OF THE INVENTION

1. *Field of the Invention*

This invention relates generally to the field of immunology and specifically to improved enhanced immunological response against human immunodeficiency virus (HIV) infection.

2. *Description of Related Art*

Acquired immune deficiency syndrome (AIDS) is a form of immunodeficiency that results from infection with a lymphocytotropic virus called human immunodeficiency virus (HIV). The World Health Organization estimates that there are currently between eight and ten million people presently infected with HIV and that this number will rise to 15-20 million by the end of this decade. By this time, the cumulative total of AIDS patients will be in the region of 16 million people which will pose an impossible burden for health care systems. Governments worldwide have allocated vast sums of money to publicize the risks of HIV infection and to educate people about the ways in which infection can be avoided, but despite all these efforts, the AIDS epidemic continues unabated. At present, even the best anti-AIDS drugs have a limited efficacy and are associated with detrimental side effects.

Furthermore, infected people, despite administration of such drugs, are still able to transmit the virus to others. Therefore, such treatment, although essential, does little to control the epidemic. As with most diseases, the

greatest hope lies in the development of an efficient, long-lasting vaccine. Knowledge of HIV and the manner in which it reacts with the host has been accumulating rapidly in the last ten years, but despite this large knowledge-base, a vaccine against HIV has yet to be developed and it seems that it will be many years before such a product is available to the general public.

The primary cellular targets for human immunodeficiency virus type 1 (HIV-1) are CD4+ lymphocytes and monocyte-derived macrophages. The lymphocyte is the major infected cell type in the blood while the macrophage is the predominant infected cell type in the brain and spinal cord. The decline in CD4+ lymphocyte concentrations in HIV-infected individuals clearly contributes to the eventual development of AIDS. Less appreciated and understood is the role of monocytes and macrophages.

Attempts to detect viral protein or nucleic acids in blood leukocytes of seropositive patients reveal a frequency of infected cells of no more than 0.001% (Harper, *et al., Proc. Natl. Acad. Sci., U.S.A.*, 83:772, 1986). Paradoxically, this low frequency of infected cells remains constant from onset of infection through late-stage disease. There is now evidence, however, that consideration of the low numbers of infected cells in the blood alone grossly underestimates the viral load in the HIV-infected patient. In certain bodily tissues, such as those of the central nervous system, lymph nodes, or lung, the frequency of HIV-infected cells may be 10,000-100,000-fold higher than that in blood. In each of these tissues, the predominant cell type infected with HIV is not the CD4+ cell, but rather the macrophage.

Numerous infected macrophages have been noted in tissues from humans that have died from HIV infection (Eilbott, *et al., Proc. Natl. Acad. Sci., USA*, 86:3337, 1989; Plata, *et al., Nature*, 328:348, 1987). In fact, the tissue macrophage may be the major cell type harboring HIV in most infected individuals. Monocyte/macrophage infection is a unifying feature of all lentiviruses, including the HIV-related lentiviruses of ungulates (visna virus,

caprine arthritis-encephalitis virus, and equine infectious anemia virus) and monkeys (simian immunodeficiency virus [SIV]). However, the importance of monocytes/macrophages for the evolution of the chronic disease caused by HIV has not heretofor been defined.

Optimally, a vaccine contains immunodominant antigens which elicit an effective immune response. At present, researchers have not been able to produce such a vaccine for AIDS, synthetic or otherwise. HIV infection can cause profound lymphopenia, primarily of the CD4 subset of T lymphocytes. Affected individuals have decreased or absent delayed-type hypersensitivity, extreme susceptibility to opportunistic infections and may acquire certain unusual malignancies such as Kaposi's sarcoma or Burkitt's lymphoma. HIV also causes polyclonal expansion of B lymphocytes, leading to hypergammaglobulinemia. Despite the marked increase in amounts of immunoglobulins in serum, affected individuals are incapable of mounting a primary immune response to newly encountered antigens. The syndrome has been recognized primarily in "at risk" groups, including homosexually active males, intravenous drug users, recipients of blood or blood products, and certain populations from Central Africa and the Caribbean. The syndrome has also been recognized in heterosexual partners of individuals in all "at risk" groups and in infants of affected mothers.

A vaccine should artificially stimulate the immune system in such a way that a subsequent entry of the live pathogenic virus into the body results in the inhibition and, preferably, the elimination of the virus and its progeny before disease occurs. One of the major problems associated with HIV is that the mechanisms of disease induction are largely unknown. Seropositive individuals can remain healthy for many years carrying very low levels of HIV within the body. During this period, the anti-HIV immune response remains very strong and yet, at a certain point in time, the virus begins to replicate rapidly and AIDS develops. Vaccines in general allow the pathogen some degree of replication, but prevent the onset of disease. However, given the

failure of the vigorous immune response to prevent the low levels of virus during the latent phase from suddenly expanding to cause disease, it has long been assumed that an AIDS vaccine must also prevent the initial establishment of infection.

An animal model which is considered to closely mimic HIV infection in humans is the monkey model which uses SIV. SIV exhibits extensive similarity to HIV in genomic organization, gene sequences, and biological properties (Desrosiers, R., *Annu. Rev. Immunol.*, 8:557, 1990; Gardner, *et al., AIDS 2* (Suppl.1):S3, 1988). Molecularly cloned $SIV_{mac}239$ causes AIDS and death in the common rhesus monkey (*Macaca mulatta*) (Kestler, *et al., Science* 248:1109, 1990). About 40% of rhesus monkeys infected with this cloned virus die with AIDS within 6 months of infection. The other 60% develop a more protracted disease course that also closely resembles AIDS in humans. Features of the AIDS-like disease include CD4+ depletion, opportunistic infections, generalized lymphoid depletion, emaciation, and a unique encephalitis, all characteristic of HIV-1-induced disease in humans. The complete genetic sequence of the $SIV_{mac}239$ clone has been determined (Regier, *et al., AIDS Res. Hum. Retroviruses*, 6:1221, 1990).

Because of the considerable similarity between SIV and HIV and the diseases which they cause in their respective hosts, a large amount of AIDS vaccine research is now carried out using the SIV_{mac} animal model system. It is perhaps ironic that the first great breakthroughs in vaccine development for HIV came using what is perhaps the simplest form of a vaccine, i.e., whole inactivated virus. In 1989 groups throughout the world, predominantly the group of Murphey-Corb in the United States, successfully protected rhesus macaques against subsequent challenge with a lethal dose of homologous virus using whole virus particles inactivated by formalin (*Science*, 246:1293, 1989). Most importantly, not only were monkeys protected from disease, but also from any measurable infection, including the absence of a virus genome in PBLs as measured by polymerase chain reaction (PCR). These results

have been repeated and substantiated in other laboratories in America and also in Europe (Carlson, *et al., AIDS Res. and Human Retroviruses*, 6:1239, 1990; Desrosiers, *et al., Proc. Natl. Acad. Sci., U.S.A.* 86:6353, 1989; Stott, *et al., Lancet*, 336:1538, 1990). These results suggested the possibility that some protein or combination of proteins in the whole virus antigen could stimulate an immune response capable of protecting against subsequent infection.

As noted above, Simian Immunodeficiency Virus (SIV)-infected macques have now proven to be a valuable animal model for the study of human AIDS. (Letvin, *et al., Science*, 230:(4721)71-73, 1985; Murphey-Corb, *et al., Nature*, 321:435-437, 1986; Zhang,, *et al., J. Infect. Dis.*, 158:1277-1286, 1988; Baskin, *et al., Vet. Pathol.*, 25:456-467, 1988). A preferred isolate for studying SIV infection is SIV/Delta. SIV/Delta was originally isolated from rhesus monkeys with an experimentally transmissible immunodeficiency disease characterized by wasting, chronic diarrhea, lymphadenopathy or lymphoid depletion, opportunistic infections and increased incidence of B-cell lymphomas. This virus is tropic for CD4 positive T-lymphocytes from rhesus monkeys and humans, but is less cytotoxic for human T-lymphocytes than HIV. Numerous inoculations of SIV/Delta have been performed in juvenile rhesus monkeys over the past several years at the Tulane Regional Primate Research Center (TRPRC) in an attempt to understand the pathogenesis of this virus. To date over 400 rhesus have been inoculated with pathogenic isolates of SIV/Delta; the current mortality in these experimental infections is greater than 90%, with 50% of the deaths occurring within 6 months post-inoculation.

Prognostic indicators of disease progression in SIV infected juvenile rhesus, often evident within the first month postinfection and several months prior to signs of clinical disease, have been identified (Zhang, *et al., supra*; Murphey-Corb, *et al., supra*) as:

1) A decline in virus-specific antibody which most often involves viral gag but not *env* determinants;

2) A selective decline in the helper-inducer T cell subset defined by dual staining with the monoclonal antibodies OKT4 and 4B4 occurring 17 to 90 days postinfection;

3) Progressive or recurrent virus-specific antigenemia which fluctuates reciprocally with antibody; and

4) Diminished primary antibody response to a test antigen (*e.g.*, tetanus toxoid).

(Baskin, *et al., Journal of the National Cancer Institute*, 77:127-139, 1986; Blanchard, *et al., Vet. Pathol.*, 24:454-456, 1987; Sharer, *et al., Annals of Neurology (Suppl.)*, 23:S108-S112, 1988; Hirsch, *et al., Nature*, 339:389-392, 1989; Baskin, *et al., Lab Invest.*, No.4, 65:400-407, 1991; Baskin, *et al., Vet. Pathol.*, 28:506-513, 1991.)

There has been a longfelt and unfulfilled need for a safe and effective AIDS vaccine. The development of a vaccine against HIV is a critical step in preventing further spread of AIDS. For safety reasons, a whole virus vaccine may not be practical in the case of HIV. The present invention, for the first time, provides a method for stimulating in a subject, a cross-protective immune responses induced by an attenuated macrophage-tropic clone of HIV, and demonstrates that rapid, protective responses appear concomitantly with broad neutralizing antibodies.

SUMMARY OF THE INVENTION

The present invention is based on the unexpected discovery that administration to a host of an attenuated immunodeficiency lentivirus, which is macrophage-tropic, as opposed to the often studied immunodeficiency lentivirus, which is only lymphocyte-tropic, induces a rapid, immune response in the host that is associated with production of broad range neutralizing antibodies. The invention shows that a live attenuated virus containing macrophage-tropic specific nucleotide sequences induces a protective immune response by 1) proper presentation of antigenic sequences to the immune system by replication in macrophages/monocytes and 2) by presentation of the correct conformational structure of specific polypeptide sequences which may be unique to macrophage-tropic proteins for induction of the appropriate immune response. Moreover, since monocyte/macrophages reside in high concentrations in mucosal surfaces, selective antigen presentation by these cells may confer a selective advantage in the induction of mucosal immunity which is necessary to block the spread of lentiviruses. Presentation of the polypeptide antigen that provides macrophage specific tropism may include a cellular determinant, therefore alloantigens may also play a role in proper induction of a protective immune response.

The invention also provides pharmaceutical compositions comprising an attenuated virus comprising a retroviral nucleotide sequence encoding a macrophage-tropic polypeptide which stimulates an immune response, in a pharmaceutically acceptable carrier. Preferably, the retrovirus nucleotide sequence encodes a macrophage-tropic polypeptide such as an HIV virus envelope (*env*) polypeptide.

BRIEF DESCRIPTION OF THE DRAWINGS

FIGURES 1A-C show a schematic illustration of the strategy for amplifying SIV envelope sequences.

FIGURES 2A-C show the detection of mutations by sequencing of PCR products from SIV infected monkey brain DNA.

FIGURE 3 shows the amino acid differences in the *env* genes of wild type and recombinant SIV.

FIGURE 4 shows the amino acid sequence of the *env* genes of wild type and recombinant SIV.

FIGURE 5 shows a flow cytometric analysis of T lymphocyte populations in the peripheral blood of monkey L238 after inoculation with SIV/17E-Cl (•, CD4+; ◦, CD8+; ▲, CD4+CD29+).

FIGURE 6 shows neutralizing antibody titers (10 $\log_{10}$) over time in sera from monkeys infected with SIV/17E-Cl (◦, L235; Δ, L238; ■, M118; ●, L652; and □, L471).

FIGURE 7 shows the avidity of serum antibodies for SIV envelope glycoproteins (◦, L235; Δ, L238; ■, M118; ●, L652; and □, L471).

FIGURE 8 shows the conformational dependence of serum antibodies to SIV envelope glycoproteins as measured by (A) native and (B) denatured viral envelope glycoprotein substrates (◦, L235; Δ, L238; ■, M118; ●, L652; and □, L471).

FIGURE 9 is a table showing antigen-specific CTL and antibody responses in monkeys immunized with *nef*-deleted monocyte and lymphocyte-tropic SIV clones.

DETAILED DESCRIPTION OF THE INVENTION

The present invention provides a method and compositions for immunological protection against the human immunodeficiency virus (HIV). As a model system, simian immunodeficiency virus (SIV) was utilized to infect macaque monkeys to show that infection with a non-pathogenic macrophage-tropic SIV provides systemic immunity by stimulation of production of neutralizing antibodies. Of major significance was the discovery that the neutralizing antibodies produced by the SIV-infected monkeys were reactive not only with the infecting virus, but several heterologous isolates of SIV as well. This model serves as the basis for a comparable method and compositions useful for inducing a protective immune response against HIV in humans.

The invention provides an immunotherapeutic method of treating a host having or at risk of having a lentivirus infection, comprising administering to the host a therapeutically effective amount of an attenuated virus comprising a retrovirus nucleotide sequence encoding a macrophage-tropic polypeptide which stimulates an immune response. An immunotherapeutic method in accordance with this invention entails the administration of the attenuated virus comprising a retrovirus nucleotide sequence encoding a macrophage-tropic polypeptide. The attenuated virus can be administered by injection or infusion, for example, prior to (prophylaxis) or following (therapy) the onset of infection with the lentivirus. The amount of attenuated virus required to induce an immune response to the lentivirus depends on such factors as the type and severity of the infection, the size and weight of the infected subject, and the effectiveness of other concomitantly employed modes of prophylaxis or therapy. This amount should be sufficient to induce an immune response in

an immunized individual which ameliorates the particular lentiviral disease as compared to the immune response in a non-immunized indiyidual.

The retrovirus nucleotide sequence encoding the macrophage-tropic polypeptide can be derived from any retrovirus and preferably is derived from a lentivirus sequence. The lentivirus family includes such viruses as human immunodeficiency virus (HIV) (including HIV type-1 and type-2), simian immunodeficiency virus (SIV), visna virus of sheep, caprine arthritis-encephalitis virus and equine infectious anemia virus. Preferably, the macrophage-tropic nucleotide sequence used in the immunotherapeutic method of the invention is derived from SIV, when the host is a non-human primate, and from HIV, when the host is a human.

The immunotherapeutic method of the invention includes a prophylactic method directed to those hosts at risk for the lentivirus infection. For example, the method is useful for humans at risk for HIV infection. A "prophylactically effective" amount of the attenuated virus comprising a retrovirus nucleotide sequence encoding a macrophage-tropic polypeptide which stimulates an immune response, for example, refers to that amount which is capable of inducing an immune response to HIV which produces some degree of protection as compared to non-immunized individuals.

Transmission of HIV occurs by at least three known routes: sexual contact, blood (or blood product) transfusion and via the placenta. Infection via blood includes transmission among intravenous drug users. Since contact with HIV does not necessarily result in symptomatic infection, as determined by seroconversion, all humans may be potentially at risk and, therefore, should be considered for prophylactic treatment by the immunotherapeutic method of the invention.

The term "therapeutically effective" means that the amount of attenuated virus administered is of sufficient quantity to increase the subject's immune

response to the virus, for example, to HIV. The dosage ranges for the administration of the virus composition are those large enough to produce the desired effect in which the HIV epitopes are focused on the surface of the APCs, thereby allowing a more efficient antigen presentation and therefore a more effective vaccination. Although not wanting to be bound by a particular theory, antigen presentation may include cellular determinants as well as HIV determinants.

The dosage should not be so large as to cause adverse side effects, such as unwanted cross-reactions, anaphylactic reactions and the like. Generally, the dosage will vary with the age, condition, sex, and extent of the disease in the patient and can be determined by one skilled in the art. The dosage can be adjusted by the individual physician in the event of any contraindications and can be readily ascertained without resort to undue experimentation. In any event, the effectiveness of treatment can be determined by monitoring the level of CD4+ T-cells in a patient. An increase or stabilization in the relative number of CD4+ cells should correlate with recovery of the patient's immune system.

The attenuated virus used in the method of the invention can be administered to a patient prior to infection with HIV (*i.e.*, prophylactically) or at any of the stages described below, after initial infection. The HIV infection may run any of the following courses: 1) approximately 15% of infected individuals have an acute illness, characterized by fever, rash, and enlarged lymph nodes and meningitis within six weeks of contact with HIV. Following this acute infection, these individuals become asymptomatic. 2) The remaining individuals with HIV infection are not symptomatic for years. 3) Some individuals develop persistent generalized lymphadenopathy (PGL), characterized by swollen lymph nodes in the neck, groin and axilla. Five to ten percent of individuals with PGL revert to an asymptomatic state. 4) Any of these individuals may develop AIDS-related complex (ARC); patients with ARC do not revert to an asymptomatic state. 5) Individuals with ARC and PGL, as well as asymptom-

atic individuals, eventually (months to years later) develop AIDS which inexorably leads to death.

The retroviral macrophage-tropic nucleotide sequence preferably encodes the envelope polypeptide including gp120 and the amino terminal 189 amino acids of gp41 (gp120/gp41(189)) (Anderson, *et al.*, *Virology*, 195:616, 1993, for nucleotide sequence). The sequence may include a fewer or greater number of nucleotide sequences, as long as the sequence still retains the macrophage-tropic activity of gp120/gp41(189). While not wishing to be bound by a particular theory, it is believed that the 3-D conformation or quaternary structure and folding of the gp120/gp41(189) amino acid sequence confers the macrophage-tropism ability to the virus.

The attenuated virus comprising a retrovirus nucleotide sequence encoding a macrophage-tropic polypeptide, such as an attenuated virus containing an HIV envelope nucleotide sequence, can be administered parenterally by injection or by gradual infusion over time. For example, the composition can be administered intravenously, intraperitoneally, intramuscularly, subcutaneously, intracavity, orally, mucosally, or transdermally.

Preparations for parenteral administration are contained in a "pharmaceutically acceptable carrier". Such carriers include sterile aqueous or non-aqueous solutions, suspensions, and emulsions. Examples of non-aqueous solvents include propylene glycol, polyethylene glycol, metabolizable oils such as, olive oil, squalene or squalane, and injectable organic esters such as ethyl oleate. Aqueous carriers include water, alcoholic/aqueous solutions, emulsions or suspensions, including saline and buffered media. Parenteral vehicles include sodium chloride solution, Ringer's dextrose, dextrose and sodium chloride, lactated Ringer's, or fixed oils. Intravenous vehicles include fluid and nutrient replenishers, electrolyte replenishers, such as those based on Ringer's dextrose, and the like. Preservatives and other additives may also

be present such as, for example, antimicrobials, anti-oxidants, chelating agents, and inert gases and the like.

Various types of attenuated viruses comprising a retrovirus nucleotide sequence encoding a macrophage-tropic polypeptide are contemplated in the method of the invention including, but not limited to, live attenuated virus (including wild-type) and live virus recombinantly engineered to contain the macrophage-tropic encoding nucleotide. Attenuation can be achieved by various methods well known to those of skill in the art, including deleting the retrovirus *nef* gene or other non-structural gene, or constructing a recombinant, infectious but non-pathogenic virus.

The method of the invention further envisions administration of nucleotide sequences encoding a macrophage-tropic polypeptide or the polypeptide itself, as synthetic peptides, DNA vaccines, natural viral products, and recombinant DNA products via various delivery vehicles. Naked DNA molecules can be directly administered *in vivo*, either by injection into muscle (Fynan, E.F., *et al., Proc. Natl. Acad. Sci. USA*, 90:11478-11482; 1993; Robinson, H.L., *et al., Vaccine*, 11:957-960, 1993; Ulmer, J.B., *et al.,, Science*, 259:1745-1749, 1993; Wang, B., *Proc. Natl. Acad. Sci. USA*, 99:4156-4160, 1990) or other tissues (Fynan, *et al., supra*), or by particle-based delivery to the epidermis (Eisenbaum, M.D., *et al., DNA and Cell Biol*, 12:791-797, 1993; Tang, *et al., Nature*, 356:152-154, 1992; Fynan, *et al., supra*). Such deliveries result in antigen expression and subsequent immune response.

Vaccination with live attenuated wild-type or recombinant virus is contemplated, either alone or in combination with adjuvant, such as aluminum hydroxide or Freund's adjuvant in a non-toxic, prophylactic or therapeutic amount. Preferably, no adjuvant is utilized, however, when administered in the form of a polypeptide, an adjuvant as described above is preferably used. An advantage of using attenuated live viral vaccine is the small amount of

material necessary to generate a strong immune response. The virus can be attenuated using methods well known in the art.

Strains of macrophage-tropic HIV useful in compositions and methods for raising neutralizing antibodies can readily be obtained from the American Type Culture Collection (Rockville, MD) or the AIDS Research and Reference Reagent Program (Rockville, MD). For instance, HIV-1jrfl, HIV-1bal, HIV-1ada, HIV-1-89.6 and HIV-1sf162 are non-limiting examples of known strains of macrophage-tropic HIV isolates that are publicly available. However, any HIV isolate that is macrophage-tropic and can stimulate production of antibodies in a patient, which cross-react with other infectious HIV, can be used as a vaccine in the practice of this invention. Methods for determining whether an HIV isolate is macrophage-tropic and methods for culturing macrophage-tropic isolates are known to those of skill in the art (see for example, Coligan, *et al., Current Protocols in Immunology*, Greene Publishing Associates, Inc. and John Wiley & Sons, Inc., 1994, Unit 12). For example, one can perform a virus titration as described in Example 1 and Table 1 of the present invention (see also McEntee, *et al., J. Gen. Virol.*, 72:317, 1991).

Delivery of macrophage-tropic specific polynucleotide can be achieved using vehicles such as a recombinant expression vector, e.g., a chimeric virus, or a colloidal dispersion system. An especially preferred colloidal dispersion system for therapeutic delivery of nucleotide sequences is the use of liposomes. Production of such vehicles are well known in the art.

Various viral vectors which can be utilized for gene therapy as taught herein include adenovirus, adeno-associated virus, herpes virus, vaccinia, or an RNA virus such as a retrovirus. Known techniques of molecular biology can be used to insert genes for antigenic epitopes for HIV virus into vectors as vehicles. Vaccinia virus has been used as one such vector. (See for example, *Current Protocols in Molecular Biology*, Ed. by F.M. Ausubel, Current Protocols, Vol. 2, §16.17, 1993). The genes for the gp 120 and amino

terminus of gp41 HIV-macrophage-tropic virus are available and can be inserted into a suitable vector using techniques well known in the art.

Examples of retroviral vectors in which a single foreign gene can be inserted include, but are not limited to: Moloney murine leukemia virus (MoMuLV), Harvey murine sarcoma virus (HaMuSV), murine mammary tumor virus (MuMTV), and Rous Sarcoma Virus (RSV). A number of additional retroviral vectors can incorporate multiple genes. All of these vectors can transfer or incorporate a gene for a selectable marker so that transduced cells can be identified and generated. By inserting a macrophage-tropic specific nucleotide sequence into the viral vector, along with another gene which encodes the ligand for a receptor on a specific target cell, for example, the vector is now target specific. Those of skill in the art will know of, or can readily ascertain without undue experimentation, specific polynucleotide sequences which can be inserted into the retroviral genome to allow target specific delivery of the retroviral vector containing the macrophage-tropic specific polynucleotide.

Since recombinant retroviruses are defective, they require assistance in order to produce infectious vector particles. This assistance can be provided, for example, by using helper cell lines that contain plasmids encoding all of the structural genes of the retrovirus under the control of regulatory sequences within the LTR. These plasmids are missing a nucleotide sequence which enables the packaging mechanism to recognize an RNA transcript for encapsidation. Helper cell lines which have deletions of the packaging signal include but are not limited to Ψ2, PA317 and PA12, for example. These cell lines produce empty virions, since no genome is packaged. If a retroviral vector is introduced into such cells in which the packaging signal is intact, but the structural genes are replaced by other genes of interest, the vector can be packaged and vector virion produced. The vector virions produced by this method can then be used to infect a tissue cell line, such as NIH 3T3 cells, to produce large quantities of chimeric retroviral virions.

Another delivery system for attenuated viruses comprising macrophage-specific polypolynucleotides or polypeptides is a colloidal dispersion system. Colloidal dispersion systems include macromolecule complexes, nanocapsules, microspheres, beads, and lipid-based systems including oil-in-water emulsions, micelles, mixed micelles, and liposomes. The preferred colloidal system of this invention is a liposome. Liposomes are artificial membrane vesicles which are useful as delivery vehicles *in vitro* and *in vivo*. It has been shown that large unilamellar vesicles (LUV), which range in size from 0.2-4.0 um can encapsulate a substantial percentage of an aqueous buffer containing large macromolecules. RNA, DNA and intact virions can be encapsulated within the aqueous interior and be delivered to cells in a biologically active form (Fraley, *et al.*, *Trends Biochem. Sci.*, 6:77, 1981). In addition to mammalian cells, liposomes have been used for delivery of polynucleotides in plant, yeast and bacterial cells. In order for a liposome to be an efficient gene transfer vehicle, the following characteristics should be present: (1) encapsulation of the genes of interest at high efficiency while not compromising their biological activity; (2) preferential and substantial binding to a target cell in comparison to non-target cells; (3) delivery of the aqueous contents of the vesicle to the target cell cytoplasm at high efficiency; and (4) accurate and effective expression of genetic information (Mannino, *et al.*, *Biotechniques*, 6:682, 1988).

Therefore, liposomes, including unilamellar bodies comprising a single lipid bilayer, can be used as vectors to deliver viral proteins, such as polypeptides specific for determining macrophage-tropism, to vaccinate against HIV virus. Such methods are taught in U.S. Patent No. 4,148,876 to Almeida, *et al.* and U.S. Patent No. 4,663,161 to Mannino, *et al.*, which are incorporated herein by reference in their entirety.

The targeting of liposomes has been classified based on anatomical and mechanistic factors. Anatomical classification is based on the level of selectivity, for example, organ-specific, cell-specific, and organelle-specific.

Mechanistic targeting can be distinguished based upon whether it is passive or active. Passive targeting utilizes the natural tendency of liposomes to distribute to cells of the reticulo-endothelial system (RES) in organs which contain sinusoidal capillaries. Active targeting, on the other hand, involves alteration of the liposome by coupling the liposome to a specific ligand such as a monoclonal antibody, sugar, glycolipid, or protein, or by changing the composition or size of the liposome in order to achieve targeting to organs and cell types other than the naturally occurring sites of localization.

The viral proteins and portions thereof, prepared as described above, may also be used in the preparation of subunit vaccines prepared by known techniques. Polypeptides displaying antigenic regions capable of eliciting protective immune response are selected and incorporated in an appropriate carrier. Alternatively, an antigenic portion of a viral protein or proteins may be incorporated into a larger protein by expression of fused proteins. The preparation of subunit vaccines for other viruses is described in various references, including Lerner, *et al.*, *Proc. Natl. Acad. Sci. USA*, 78:3403, 1981 and Bhatanagar, *et al.*, *Proc. Natl. Acad. Sci. USA*, 79:4400, 1982. See also, U.S. Patent Nos. 4,565,697 (where a naturally-derived viral protein is incorporated into a vaccine composition); 4,528,217 and 4,575,495 (where synthetic peptides forming a portion of a viral protein are incorporated into a vaccine composition). Other methods for forming vaccines employing only a portion of the viral proteins are described in U.S. Patent Nos. 4,552,757; 4,552,758; and 4,593,002. The relevant portions of each of these patents are incorporated herein by reference.

Such vaccines are useful for raising an immune response against HIV, for example a protective antibody titer, in humans susceptible to the virus. The attenuated viruses or vehicles containing macrophage-tropic specific sequences, prepared as described above, may be administered in any conventional manner, including nasally, subcutaneously, or intramuscularly.

Adjuvants will also find use with subcutaneous and intramuscular injection of completely inactivated vaccines to enhance the immune response.

Live attenuated viruses can also be incorporated into immunostimulating complexes (ISCOM) for use as a vaccine using methods well known in the art. SIV recombinant vaccine containing macrophage-tropic specific sequences, gp120 and the amino terminus of gp41 are shown in the present invention to raise high neutralization antibody titers after about 10 $TCID_{50}$ (tissue culture infectious dose; grown in primary rhesus peripheral blood mononuclear cells and injected into rhesus macaque monkeys (EXAMPLE 5). The recombinant SIV or HIV can be incorporated into ISCOM particles which are useful for prophylactic or therapeutic vaccination against SIV or HIV infection, respectively.

The presentation of viral protein antigens in ISCOM particles has three main advantages: 1) no replicating viral nucleic acid is introduced into the host, 2) high levels of neutralizing antibodies are achieved, and 3) a cellular immunity is evoked, including cytotoxic T-cells induced under restriction of MHC class II. The methodology for making ISCOM vaccines is well known in the art (B. Morein, *et al., Nature*, 308:457-60, 1984).

In addition, the invention provides a novel pharmaceutical composition which may be useful in the immunotherapeutic method of the invention, for example. The pharmaceutical composition comprises an attenuated virus comprising a retrovirus nucleotide sequence encoding a macrophage-tropic polypeptide which stimulates an immune response, in a pharmaceutically acceptable carrier, as described above. The retroviral nucleotide sequence encoding a macrophage-tropic polypeptide preferably encodes a virus envelope (*env*) polypeptide, most preferably, the HIV envelope. The nucleotide sequence encoding the HIV envelope protein, for example, is in operable linkage in a lentivirus genome, thereby allowing efficient transcription and translation of the envelope.

The pharmaceutical composition may comprise a recombinant chimeric lentivirus which is macrophage-tropic, in a pharmaceutically acceptable carrier. The composition comprises a recombinant chimera containing at least a gene encoding the lentivirus envelope protein. Preferably the chimera includes a gene that encodes an HIV macrophage-tropic envelope protein which replaces an SIV envelope encoding gene, in an SIV genome. The HIV envelope gene is inserted in operable linkage in the SIV genome so that it is efficiently transcribed and translated. The SIV background genome may be macrophage-tropic or it may be lymphocyte-tropic. Alternatively, the pharmaceutical composition may include a recombinant virus which includes a macrophage-tropic HIV envelope gene inserted in a lymphocyte-tropic or other macrophage-tropic HIV genome in operable linkage.

Alternatively, a pharmaceutical composition of the invention includes vehicles for delivery of nucleotide sequences encoding a macrophage-tropic polypeptide or the polypeptide itself, such as synthetic peptides, DNA vaccines, natural viral products, and recombinant DNA products, in a pharmaceutically acceptable carrier. Such vehicles may include, but are not limited to, RNA and DNA virus vectors and liposomes.

The following examples are intended to illustrate but not limit the invention. While they are typical of those that might be used, other procedures known to those skilled in the art may alternatively be used.

EXAMPLE 1

MATERIALS and METHODS

Viruses and Cell Culture

The molecular clones of $SIV_{mac}251$ and $SIV_{mac}239$ (Naidu, *et al., J. Virol.*, 62:4691-4696, 1988) were obtained from Dr. Ronald Desrosiers. The complete nucleotide sequences of the viruses have been previously reported (Regier and Desrosiers, *AIDS Res. Hum. Retroviruses*, 6:1221-1232, 1990;

Chakrabarti, *et al., Nature (London)*, 328:543-547, 1987; Franchini, *et al., Nature (London)*, 328:539-542, 1987). $SIV_{mac}239$ was serially passaged in monkeys to obtain $SIV_{mac}239$/R71-BR from the brain of macaque R71 (Sharma, *et al., J. Virol.*, 66:3550-3556, 1992a). This virus, hereafter referred to as R71 virus, was inoculated intracerebrally into the brain of macaque 17E and gave rise to $SIV_{mac}239$/17E-BR, referred to as 17E virus. The HUT-78 and C8166 (Salahuddin, *et al., Blood*, 68:281-284, 1983) T-cell lines, and the CEMx174 T-cell/B-cell fusion cell line (Salter, *et al., Immunogenetics*, 21:235-246, 1985) and the U937 monocyte/macrophage cell line were maintained according to standard culture technique, using RPMI medium with 10% fetal bovine serum (GIBCO) and 2 mM glutamine (GIBCO). Primary rhesus macaque peripheral blood mononuclear cells PBMCs were isolated and maintained using standard techniques, as previously described (Sharma, *et al., supra,*).

Amplification of SIV DNA

PCR was used to directly amplify the SIV DNA from frozen brain tissue of the R71 and 17E monkeys. Tissues were homogenized individually with a frozen mortar and pestle into a suspension using a solution of 1% sodium dodecyl sulfate (SDS), 100 mM NaCl, 50 mM Tris-HCl (pH 7.5), 1mM EDTA, and 0.1 mg/ml proteinase K (Boehringer-Mannheim). Subsequent DNA isolation was carried out as previously described (Sharma, *et al., supra*). To control for contamination, parallel samples of uninfected HUT-78 cells were lysed similarly and carried through subsequent procedures. PCR was done using standard conditions as previously described (Sharma, *et al., supra*) with several modifications. Primers were synthesized complementary to $SIV_{mac}239$ sequences homologous to conserved regions of HIV-1 (Starcich, *et al., Cell*, 45:637-648, 1986; Hahn, *et al., Nature (London)*, 232:1548-1553, 1986; Alizon, *et al., Cell*, 46:63-74, 1986; Benn, *et al., Science*, 230:949-951, 1985) on an Applied Biosystems DNA synthesizer. Overlapping fragments of 1.0 to 1.3 kilobases (kb) were amplified. An initial amplification for R71 DNA was first carried out using the following primer set 5'-AAGCTTGGATCCGCATGC-

TATAACACATGCTATTGT-3'(5.1) (SEQ ID NO:1) and 5'-AAGCTTGAATTC-GGAGGTTCTTTGTTCCCCAGACGG-3'(3.1) (SEQ ID NO:2), which are complementary to bases 6446 to 6469 and 8379 to 8402 of $SIV_{mac}239$, respectively (Regier and Desrosiers, *supra*). Both primers have 12 non-homologous bases at the 5' end. Another initial reaction was carried out using the following primer set 5'GCTTTGCTTAGATGTAATGACAC-3' (235) (SEQ ID NO: 3) and 5'-TGGATATGGGTCTGCTGGAA-3' (443) (SEQ ID NO:4), which are complementary to bases 7318 to 7340 and 8797 to 8816, respectively. Samples were subjected to 30 cycles of 1 min of denaturation at 94°, 1 minute of annealing at 55°, and either 2 minutes of extension at 72° for 5.1 to 3.1 or for 1 minute 30 seconds for 235 to 443. Ten microliters of these reactions was used as starting material for secondary reactions (200 μl total volume) using the following second primer sets 5'-AAGCTTGGATCCGG-CTTGGGGATATGTTATGAGCAA-3' (5.2) (SEQ ID NO:5) and 5'-CCTGGTC-TTCTACATTTCATTG-3' (388) (SEQ ID NO:6), which are complementary to bases 6512 to 6535 and 7526 to 7547, respectively; 5'-TGCACAAGGATGAT-GGAGACA-3' (279) (SEQ ID NO: 7) and 3.1, with 279 being complementary to bases 7390 to 7410; and 5'-CTCTATCGATTGGAATTGGGAG-3' (239) (SEQ ID NO: 8) and 443, with 239 being complementary to bases 8068 to 8089. Primer 5.2 also has 12 non-homologous bases on the 5' end. The 5.1 to 3.1 reaction was used as starting material for reactions with primers 5.2 and 388 as well as reactions with primers 235 and 3.2 The 235 to 443 reaction was used as starting material for reactions with primers 279 to 3.1, as well as primers 239 and 443. For amplification of 17E SIV DNA, the initial reactions were done with primer set 5.1/388 and also with primer set 235/443. The 5.1/388 reaction was used as starting material for reactions with primer set 5.2/388. The 235/443 reaction was used for starting material for reactions with primer set 279/3.1, as well as primer set 239/443. The primers and amplified products for R71 are shown schematically in FIGURE 1A.

The 17E envelope (along with *tat* and *rev* 5' ORF) was amplified by PCR from DNA isolated from brain homogenates that were co-cultivated with primary

monkey macrophages. Sequences from the *Eco*47III restriction site (bp 6351) to the *Nhe*I site (bp8742) were amplified by a single PCR reaction (described above). The 5' primer was made complementary to bases 6343 to 6362 (481) 5'-AAGCTTGGATCCCTCCAACGAGCGCTCTTAAT 3' (SEQ ID NO: 9) and the 3' primer was made complementary to bases 8743 to 8763 (407) 5'-AAGCTTGGATCCCCCCTGCCTTAACTTAGCTAG 3' (SEQ ID NO: 10). Both primers have *Hind*III sites at their 5' ends to facilitate intermediate molecular cloning and nucleotide sequence analyses. The *Eco*74III to *Nhe*I fragment from the PCR amplification was cloned into the $SIV_{mac}239$ molecular clone (p239).

Direct Sequencing of Amplified DNA

PCR products were sequenced directly using previously described methods (Kusukawa, *et al.*, *BioTechniques*, 9:66-72, 1990). Briefly, products were precipitated by adding 0.6 volume of 20% polyethylene glycol and 2.5 M NaCl, which left the majority of the PCR primers in solution. Primers used for sequence analysis were end-labeled with ^{32}P, annealed to the PCR products by boiling of the labeled primer and template followed by rapid cooling. Klenow (Pharmacia) was added, and this reaction was added to mixtures of cold deoxy and dideoxy nucleotides. Since the amplified DNA is expected to be heterogeneous because of anticipated mutations of the virus in the brain and because of possible errors caused by *Taq* polymerase, a number of precautions were taken to distinguish between the two sources of mutations. Three individual PCR products were synthesized and analyzed. Sequence determination of several regions of R71 and 17E were performed using two different techniques: automated sequencing using the Applied Biosystems model 373A automated cycle sequence as well as the method described above. In addition, control sequencing reactions were carried out with the PCR products from reactions with cloned $SIV_{mac}239$ DNA. Molar quantities of cloned DNA from $SIV_{mac}239$ and $SIV_{mac}251$ were mixed together to generate mixed populations of products with defined sequences. Primers used for this test were complementary to regions of the LTR of $SIV_{mac}239$ and $SIV_{mac}251$

which were identical. They were 5'-AAGCTTCTCGAGCATTTGGC-TGGCTATGGAAATTAG-3' (SEQ ID NO: 11) and 5'-AAGTTGGATCCCTCTA-CCTGCTAGTGCTG-3' (SEQ ID NO: 12), which are complementary to bases 124 to 147 and 567 to 584, respectively, of $SIV_{mac}239$. PCR was done using standard reaction conditions, 25 cycles of 1 minute of denaturation at 94°, 1 minute of annealing at 55°, and 1 minute of extension at 72° with either 10 ng of $SIV_{mac}251$ DNA, 5 ng of $SIV_{mac}239$ DNA plus 5 ng of $SIV_{mac}251$ DNA or 1 ng of $SIV_{mac}239$ DNA plus 9ng of $SIV_{mac}251$ DNA as plates. The PCR product was prepared and sequenced as above.

Subcloning of Envelope Sequences

Envelope sequences from R71 were subcloned into pBS- (Stratagene) cloning vector. The nucleotide sequence of these clones was determined to identify the nucleotide changes in individual *env* genes. The PCR products were generated using an overlapping mutagenesis technique (Higuchi, *et al.*, *Academic Press*, pp.177-183, 1990) which incorporates a single base pair silent mutation that abrogated recognition of the *Tth*111I site at nucleotide 8315 by that restriction enzyme. The product of this mutagenesis was confirmed both by the loss of recognition by *Tth*111I as well as by nucleotide sequence analysis. The mutagenesis of the *Tth* 111I site was done to facilitate the construction of recombinant viruses (see below). Two initial reactions were done using the following primer sets 5'-AAGCTTGAATTCGCA-TCAGCAAAAGTAGACATGG-3; (406) (SEQ ID NO: 13) and 5'-CTCTTGAC-CACATCCAACAGCTG-3' (408) (SEQ ID NO: 14), which were complementary to bases 7015 to 7036 and 8302 to 8324, respectively; and 5'-CAGCTGTT-GGATGTGGTCAAGAG-3' (409) (SEQ ID NO:15) and 5'-AAGCTTGGATCC-CCCCTGCCTTAACTTAGCTAG-3' (407) (SEQ ID NO:16), which are complementary to bases 8302 to 8324 and 8743 to 8763, respectively. The bases in bold type in 408 and 409 are the mismatches between these primers and $SIV_{mac}239$, creating silent mutation. Primers 406 and 407 have 12 nonhomologous bases that contain the recognition sequences for *Eco*RI and *Bam*HI, respectively, for cloning. These initial reactions were carried out using

standard conditions and 25 cycles of 1 minute of denaturation at 94°, 1 minute of annealing at 60°, and 1 minute of extension at 72°. A second reaction was initiated using 15μl of the 406 to 408 product and 5 μl of the 409 to 407 product as starting material, plus more of primer 406 and primer 407 to a final concentration of 1 μM. Using standard conditions, reactions were subjected to 30 cycles of 1 minute of denaturation at 94°, 1 minute of annealing at 60°, and 1 minute 45 seconds of extension at 72°. Primers 408 and 409 are complementary to each other and therefore the PCR products were expected to hybridize and subsequently be filled in by *Taq* polymerase as shown in FIGURE 2. PCR products were digested with *Eco*RI and *Bam*HI and subcloned into the pBS-vector. Subclones were sequenced using the dideoxy method (Sanger, *et al., Proc. Natl. Acad. Sci. USA*, 74:5463-5467, 1977).

Construction of Recombinant Viruses

The $SIV_{mac}239$ clone was digested with *Eco*RI, and the complete infectious provirus and flanking cellular DNA were subcloned into pUC19. The plasmid, p239, which is infectious, was digested with *Nhe*I and *Tth*111I and purified by agarose gel electrophoresis. Envelope subclones in pBS were digested with *Nhe*I and *Tth*111I, purified by agarose gel electrophoresis, and ligated into the p239 provirus to create p239-R71-1-1, p239-R71-2, p239-R71-10, p239-R71-13, and p239-R71-14. Two control recombinants were made placing either the *env* region from $SIV_{mac}239$ or $SIV_{mac}251$ into the *Tth*111I and *Nhe*I sites of p239, using the same procedures as with the R71 recombinants.

Another set of recombinant clones were constructed using the nucleotide sequence amplified from the 17E virus that included (5' end at bp 6351 *Eco*47III) part of *tat*, all of the *rev* 5'ORF, and the *env* gene to the *Nhe*I site (bp 8742). This fragment was cloned into these unique sites in p239. Eight clones were tested for infectivity by electroporation into CEMx174 cells. Clone 17E-2, 17E-3, 17E-5, 17E-6, and 17E-8 were infectious. The complete nucleotide sequence of the inserted fragment in clones 17E-2 and 17E-3 has been determined. The cellular-tropism of these infectious clones was

compared to the parental virus $SIV_{mac}239$ and $SIV_{mac}17E$ brain by titration of the virus stocks in CEMx174 and primary rhesus macrophages (McEntee, *et al., J. Gen. Virol.*, 72:317-324, 1991).

Transfection of Recombinant Viruses

Five to 10 μg of p239 or recombinant plasmids were introduced in four separate experiments into either C8166 (one experiment), CEMx174 (one experiment), or primary rhesus macaque PBMCs (two experiments) using the DEAE-dextran method with and without DMSO shock (Lopata, *et al., Nucleic Acids Res.*, 12:5707-5717, 1984). Cultures incubated either alone, or with the addition of fresh cells of the same type immediately or 48 hours later were monitored for cell fusion cytopathic effect (CPE) at 2 to 3 day intervals for 2 to 3 weeks. Appearance of CPE in p239 and recombinant controls transfected cultures was usually observed between 7 and 10 days after transfection. CEMx174 cells were added to PBMC cultures on Day 7 to amplify virus production. Additional experiments were done by electroporation, using the Bio-Rad Gene Pulser. Conditions used for PBMCs were according to manufacturer's specifications, with a pulse of 300v and 500 μF. CEMx174, HUT-78, and U937 cells were electroporated according to manufacturer's specifications also, with 200v and 960 μF. Two electroporation experiments were done using PBMCs and CEMx174 cells and one each in HUT-78 and U937 cells. Cells were assayed for viral replication, a subset of cultures from different experiments was assayed for SIV p27 levels in the supernatant using a commercial immunoassay (Abbott) for detection of p24 of HIV. Here, p239, as well as the control recombinants were positive within 5-10 days. Recombinants were monitored for CPE for 2 to 3 weeks.

EXAMPLE 2

EXAMINATION OF SIV DNA FROM INFECTED BRAIN TISSUE

In order to examine the *env* sequence of the SIV virus present in the brains of monkey with CNS disease without the selective pressure of culturing *in vitro*, DNA from brain was amplified by PCR and the nucleotide sequences of the fragments were determined. Cellular DNAs obtained from the brains of monkeys R71 and 17E, as well as from uninfected HUT-78 cells, were used for PCR amplification of envelope sequences as described in Example 1 and FIGURE 1. FIGURE 1 shows the strategy for amplifying SIV envelope sequences. Primers are shown as arrows, primer number is next to arrow, and direction of arrow indicates 5' to 3' orientation. FIGURE 1 (A): Generation of initial PCR products with the terminal nucleotide positions indicated below, and above shows position relative to SIV *env* gene below. FIGURE 1 (B): Second round of PCR done with internal primers to generate the products used for sequencing. FIGURE 1 (C): R71 *env* regions were synthesized for subcloning by mutation of one of the two *Tth*111I sites in the SIV *env*. The "X" in primers 408 and 409 indicates a point mutation which alters the second *Tth*111I. This base pair change does not alter the amino acid sequence. Primers 408 and 409 are complementary, so the two initial products shown, containing the mutation, will hybridize to each other in a second reaction. This second reaction gives the product shown, from nucleotide 7015 to 8763. The jagged lines indicate nonhomologous sequences which contain restriction enzyme sites to facilitate cloning.

No PCR amplification products were obtained from uninfected HUT-78 DNA samples. The primers used for PCR were made complementary to *env* regions that would be expected to be conserved by analogy to the HIV envelope. Overlapping fragments were generated to determine if in fact the regions to which the primers bound were conserved. Nucleotide sequence analysis showed that the internal primers were in fact complementary to sequences that had remained identical to $SIV_{mac}239$ (see below). The

population of envelope genes present in the brain was amplified by PCR, and the nucleotide sequence of the amplified DNA was determined to identify nucleotides that were distinct from those of $SIV_{mac}239$.

EXAMPLE 3

ANALYSIS OF SIV NUCLEOTIDE SEQUENCES

While some nucleotide substitutions were homogeneous, meaning that the mutant base had completely replaced the "wild-type" base (FIGURE 2A), several sites were found to contain two bases (FIGURE 2B). Because this could have been an artifact of the sequencing techniques or *Taq* polymerase, several steps were taken to verify that these sequence changes originated in the brain. First, PCR amplification and sequence determinations were performed on $SIV_{mac}239$ *env* gene sequences to test for sequence specific artifacts (FIGURES 2A and 2B, reaction 5). Second, the PCR product was sequenced on the opposite strand by the same technique or by automated sequencing (see EXAMPLE 1). Third, an amplified product from two to three unique PCR reactions was sequenced (FIGURES 2A and 2B). This allowed the determination of the origin of the altered base, either an error in *Taq* polymerase incorporation or an actual change in the SIV DNA isolated from brain.

A ^{32}P end-labeled primer complementary to nucleotides (nt) 6917 to 6938 was used for reactions in FIGURE 2A and a similarly labeled oligonucleotide complementary to bases 7917 to 7938 was used for reactions in FIGURE 2B. Unique PCR products from R71 DNA (reactions 1 and 2), unique products from 17E DNA (reactions 3 and 4), and unique products from control cloned SIV_{mac}DNA (reaction 5) are shown in FIGURE 2A and FIGURE 2B. Sequencing lanes are in the following order: A,C,G, and T, and the sequence is shown on the left of each reaction. The oligonucleotide in (B) is complementary to the plus-strand and gives minus-strand sequences. The autoradiograph that is shown was turned over to yield the sequences of the plus-strand. The

complete A-G transition at nt 7025 is shown in (A), which is predicted to change lysine 141 to arginine. A mixture of both a T and a C residue at no 7864 is shown in (B), which encodes either serine or proline at the position of proline 421. FIGURE 2C is a control experiment in which direct sequencing was carried out on PCR products from the LTR of cloned SIV_{mac}251 DNA (reaction 1), cloned SIV_{mac}239 DNA and cloned SIV_{mac} 251 DNA present in equal amounts in the initial PCR reaction (reaction 2), and cloned SIV_{mac}239 DNA and cloned SIV_{mac}251 DNA (reaction 3) present in a 1:9 molar ratio as starting material (reaction 3). The appearance of the C residue in reaction 2 at the position indicated by the arrow is due to the SIV_{mac}239 sequence, and is barely detectable in reaction 3.

To test the possibility that a heterogeneous population of DNAs in the reaction would lead to detection of multiple bases at a single site, mixtures of cloned DNA having known nucleotide differences by PCR were amplified and the products sequenced directly. Two bands were found at sites where the cloned DNAs differed (FIGURE 2C). While use of these techniques did not permit precise quantitation of the percentages of the variants in the population of starting material, control experiments indicated that a variant nucleotide was detectable if it comprised 10% or greater of the total population (FIGURE 2C).

EXAMPLE 4

AMINO ACID CHANGES IN SIV ENVELOPE POLYPEPTIDE

Using the criteria described above in Example 3, the amino acid changes found in the R71 *env* in FIGURES 3 and 4 are shown. A diagram of the envelope gene of SIV_{mac}239 is shown to scale with the position of HIV-1 variable regions and the CD4 binding domain. The *Tth*111I and the *Nhe*I sites used for subcloning envelope regions as well as the gp 120-gp32 cleavage site are shown with the amino acid position indicated beneath (FIGURE 3). The wild-type SIV_{mac}239 amino acids which are altered in R71 are given for

reference, with their positions alternating between above and below the one letter amino acid code. Mutated amino acids determined by direct sequencing of the PCR products from R71 brain DNA are as shown, with boxed amino acids indicating a complete loss of the nucleotide found in $SIV_{mac}239$ that was predicted to give the "wild-type" amino acid. Mutations not boxed indicate a mixture of the mutant and wild-type amino acids at that site. The asterisks indicate silent mutations. Results from sequencing individual clones from a different PCR reaction are shown (R71 to R71-14). The (-) indicates predicted amino acids identical to $SIV_{mac}239$, yet different from the R71 population. Changes here are also indicated by the one letter abbreviation. The ($) symbol in R71-2 indicates mutations to stop codons. Data from direct sequencing of 17E brain DNA are shown in the same manner as the R71 direct sequencing.

The complete amino acid sequence of the *env* gene of SIV_{mac} from amino acids 1-738 is listed in FIGURE 4, with the changes preser ⁻ the brain isolates and molecular clones. An asterisk indicates a silent m..ation, while a dash indicates no change from the $SIV_{mac}239$ sequence. The amino acid changes found to contribute to macrophage-tropism in $SIV_{mac}239/316$ are shown in the last lines marked 316 (Mori, *et al., J. Virol.*, 66:2067, 1992). Regions analogous to the HIV-1 variable regions are bracketed (V1-V5) and the surface membrane protein-transmembrane protein cleavage site is indicated by an arrow.

Only a limited number of nucleotide changes were found in the R71 envelope sequence when compared to $SIV_{mac}239$. These nucleotide changes were located throughout the envelope gene. For further analysis of mutations, the envelope region from nucleotides (nt) 7015 to 8763 was amplified in a separate reaction, as shown in FIGURE 1B. This approach was also employed to facilitate the construction of recombinant clones of $SIV_{mac}239$ with R71 envelope sequences. The R71 envelope sequences were inserted between the unique *Nhe*I site at nt 8746 and the *Tth*111I site at nt 7034, as

discussed below. These fragments were first subcloned into pBS (Stratagene), sequenced using the Sanger dideoxy method (FIGURE 3:R71-1,2,10,13 and 14). These results showed that closely related variants were present within a single PCR product. Most, but not all, of the changes identified in the population were found in each individual envelope clone. In addition, there were some unique changes (i.e., Y183C in R71-10 and R338K in R71-2); however, it is impossible to determine if these unique mutations occurred *in vivo* or during amplification *in vitro*. Recombinant viruses were subsequently generated using the infectious molecular clone of $SIV_{mac}239$ and the PCR derived envelope sequences of R71 from the *Tth*111I site to the *Nhe* site. The recombinant DNAs were transfected into primary rhesus PBMCs as well as four cell lines. However, none of these recombinants produced infectious virus. The control plasmid, $SIV_{mac}239$(p239), as well as other recombinant p239 viruses, replicated in the PBMCs and the cell lines when tested in parallel.

To determine whether the changes found in macaque R71 were stable and maintained in the next *in vivo* passage in monkey brain, DNA was isolated from the brain of macaque 17E and amplified by PCR to synthesize overlapping envelope products. The amino acid changes found in the 17E brain DNA were very similar to those found in R71 (FIGURES 3 and 4). The amino acid sequences that have been found to contribute to macrophage-tropism in $SIV_{mac}239$ have been included in FIGURE 4 ($SIV_{mac}239$/316 from Mori, *et al., supra*).

The *env* gene from the DNA obtained from macaque 17E was amplified from the *Eco*47III (bp 6351) site within the 5'ORF of the *tat* gene to the *Nhe*I site (bp8742). This DNA fragment was inserted in the $SIV_{mac}239$ infectious clone (p239) and transfected into CEMx174 cells (lymphocyte cell line). Five infectious recombinant viruses were obtained cloned 17E-2, 17E-3, 17E-5, 17E-6, and 17E-8. Two of these clones have been characterized further. The complete nucleotide sequence of the *Eco*47III to *Nhe*I site of clones 17E-2

and 17E-3 was determined. The clones had identical nucleotide sequences. The amino acid changes found in clones 17E-2 and 17E-3 are shown in FIGURE 3 and 4. Most of the amino acid changes are in common with those identified in R71 and 17E described above. One base pair change was found in the *tat* and *rev* 5'ORFs. This change does not alter the amino acid sequence of *rev* but it causes a conservative change in the *tat* protein located outside any functional domains that has been identified.

EXAMPLE 5

INFECTION OF RHESUS MACAQUE MONKEYS WITH RECOMBINANT SIV

As a model system, infection with the macrophage-tropic clone SIV/17E-Cl was used in the following Examples. SIV/17E-Cl contains the gp120 and a portion of the gp41 sequences (to amino acid 730) from the macrophage-tropic/neurotropic virus strain SIV/17E-Br in the background of the infectious molecular clone $SIV_{mac}239$, which replicates poorly in monocyte/macrophages (H. Kestler, *et al.*, *Science*, 248:1190, 1990). A comparison of the cell tropism of the two parental viruses and the recombinant molecular clone (Table 1) showed that the *env* sequences from the macrophage-tropic strain SIV/17E-Br conferred a tropism for macrophages to $SIV_{mac}239$. Although the recombinant clone replicated to a comparable level as the parental virus (SIV/7E-Br) in primary rhesus macrophages *in vitro*, it replicated relatively poorly in primary peripheral blood lymphocytes.

TABLE 1

TITRATION OF VIRUSES IN A LYMPHOCYTE CELL LINE AND PRIMARY RHESUS PBLs AND MACROPHAGES

Virus	CEMx174[a]	Rhesus PBL	Rhesus Macrophages
$SIV_{mac}239$	10^5	10^4	10^1
SIV/17E-Br	10^5	10^4	10^4
SIV/17E-Cl	10^3	10^2	10^4

[a]$TCID_{50}$ produced in the particular cell type and titrated in CEMX174 cells.

(The Tulane Regional Primate Research Center takes responsibility for humane care and use of laboratory animals used in projects awarded by the Public Health Service. The present invention complied with the Principles for Use of Animals, *The Guide for the Care and Use of Laboratory Animals*, the Provisions of the Animal Welfare Act, and other applicable laws and regulations. The Center's statement of assurance is on file with the PHS Office for Protection from Research Risks (Assurance number A3701-01). This facility is accredited by the American Association for Accreditation of Laboratory Animal Care. Animals are anesthetized with ketamine prior to all procedures that require the removal of animals from their cages. No restraining devices are necessary during these procedures. When necessary, moribund animals are euthanized by intravenous inoculation of a lethal dose of sodium pentabarbitol).

Eight rhesus macaques were inoculated intravenously with $10TCID_{50}$ (Tissue Culture Infections Dose) SIV/17E-Cl that had been grown in primary rhesus peripheral blood mononuclear cells. Monkeys were inoculated intravenously with cell culture supernatants from rhesus monkey peripheral blood mononuclear cells infected with SIV/17E-Cl while under sedation with ketaset

anesthesia using a 23 gauge butterfly into the saphenous vein. This material was prepared and frozen at 70°C prior to use. Monkeys were monitored for signs of infection and disease by virus culture, complete physical examination, complete blood counts, enumeration of lymphocyte subsets by flow cytometry, seroconversion, and polymerase chain amplification of viral DNA in PBMC's at weekly intervals for the first month, and every other week thereafter. The monkeys became persistently infected, but no change in physical or immunological appearance was observed.

All monkeys seroconverted and became persistently PCR positive following inoculations determined by the following method. Peripheral blood mononuclear cells (PBMC) were isolated from heparinized blood on ficoll-hypaque density gradients. Where indicated, mononuclear cells were isolated from lymph node biopsies by gentle teasing of biopsied tissue. Cells were washed with RPMI culture medium prior to lysis and the DNA purified from detergent disrupted cells by solvent extraction followed by spooling onto a glass rod. Identification of viral sequences was performed using a nested PCR reaction using conserved sequences in the viral LTR. Each PCR reaction mixture contained 10 mM Tris-HCL, pH 9.0 at 25°, 50 mM KCl, 1.75 mM $MgCl_2$, 0.01% (w.v) gelatin, 2 mM dNTP, 20 pM 5' and 3' oligonucleotide primers, and 2.5 U *Taq* polymerase (Promega). One microgram of DNA was then amplified by 30 cycles in a DNA Thermocycler (Perkin-Elmer Corp., Norwalk, CT). The first cycle consisted of denaturation at 94° for 1 minute, annealing at 55° for 1 minute, and extension at 72° for 1 minute plus 10 seconds for each 30 cycles. A second nested round was denatured for 1 minute at 94°, annealed at 45° for 1 minute, and extended at 60° for 1 minute plus 10 seconds for each of 30 cycles. Fifteen percent of the amplified product was then electrophoresed through a 2% agarose gel and visualized by ethidium bromide staining. The sequences of the LTR-specific primer pairs used in the first round were (5') 5'-ATAGTTGCAGTACATGTGGCTAGTG-3' (SEQ ID NO:17) and (3') 5'-TCTCTGCCTCTTTCTCTGTAATAGAC-3' (SEQ ID NO:18) and, for the second round, (5') 5'-AGGCAGAAAGGGTCCTAC-

AGACCAGGGT-3' (SEQ ID NO:19) and: (3') 5'-AGGCAGAAAGGGTCCTACAGACCAGGGT-3' (SEQ ID NO:20). To confirm the specificity of the amplified product, fragments were Southern blotted after electrophoresis to a Magna NT Nylon Transfer Membrane (MSI, Westboro, MA) and hybridized to a ^{32}P-labeled oligonucleotide complementary to sequences within the primers used in the amplification reaction. The sequence of the oligonucleotide probe was (5') 5'-AGCAGGTAGAGCCTGGGTGTTC-3' (SEQ ID NO:21).

Five monkeys were serially monitored for infectious virus by culture of both peripheral blood lymphocytes and macrophages (Table 2). At early times (7-14 days) postinoculation, virus could be cultured from all animals from both populations; thereafter, virus could not be cultured from lymphocytes and only periodically from macrophages. However, macrophage cultures were persistently positive by PCR analysis for SIV/17E-Cl envelope sequences for up to 55 weeks after inoculation.

TABLE 2

Infection Animal	Cells	DAYS AFTER INOCULATION 7	21	28	195	218	258
L235	Macrophage[a]	+	-	-	-	-	-
	PBL[b]	+	-	-	-	-	-
L238	Macrophage	+	-	-	-	-	-
	PBL	+	-	-	-	-	-
L471	Macrophage	+	-	+	-	+	-
	PBL	+	-	-	-	-	-
L652	Macrophage	+	-	+	-	+	-
	PBL	+	-	-	-	-	-
M118	Macrophage	+	-	+	-	+	-
	PBL	+	-	-	-	-	-

[a]Macrophages were cultured from the peripheral blood and virus was assayed both by titration of supernatants and by the co-cultivation with CEMx174 cells at 14 days after isolation.

[b]Lymphocytes were cultured from the peripheral blood and virus was assayed both by titration of supernatants and by the development of virus induced CPE (cytopathic effect) at 7 and 14 days after isolation.

The development of AIDS-like disease was monitored in all 8 SIV/17E-Cl-infected monkeys by detection of viral p26 serum and by flow cytometric measurement of changes in T lymphocyte populations in the peripheral blood. Of particular interest was either a reciprocal decline in CD4+ (helper) and an increase in CD8+ (suppressor) T lymphocytes, or a selective decline in the CD4+CD29+ (helper-inducer) T lymphocyte population. A selective decline in CD4+CD29+ T lymphocytes has been shown to be a reliable early indicator of disease progression in monkeys infected with the pathogenic isolate SIV/DeltaB670 (M. Murphey-Corb, *et al.*, *Science*, 246:1293, 1989). The percentages of T lymphocyte populations observed over time after infection of macaques with the lymphocyte-tropic strain $SIV_{mac}239$ (H. Kestler, *et al.*, *supra*; D.P. Sharma, *et al.*, *J. Inf. Dis.* 166:738, 1992), no significant changes

in T lymphocyte populations were observed, and none of the SIV/17E-Cl-infected animals had detectable SIV p26 antigenemia at any time postinfection. This low level of virus replication in infected animals with SIV/17E-Cl probably reflects the cell tropism of the virus and the pattern of replication of SIV in macrophages *in vivo*. Five monkeys have been followed for 15 months and 4 macaques (2 challenged, 2 unchallenged) have become virus negative by culture and intermittently negative by PCR analysis of PBMC's.

EXAMPLE 6

DEVELOPMENT OF NEUTRALIZING ANTIBODY RESPONSES

By 14 days postinoculation, all 5 monkeys monitored serially had neutralizing antibodies directed against the infecting strain, SIV/17E-Cl. FIGURE 6 shows neutralizing antibody titers ($10\log_{10}$) over time in sera from monkeys infected with SIV/17E-Cl. Neutralization of SIV/17E-Cl was performed in 96-well tissue culture plates containing RPMI supplemented with 10% fetal bovine serum. Five-fold serial dilutions of plasma (heat inactivated at 56°C and clarified by centrifugation) were added to each, each with 10-100$TCID_{50}$ of virus and incubated 1 hour at 37°C. $1x10^6$ CEMx174 cells were added to each well and the development of CPE was recorded at 7 days. The 50% neutralization endpoint was calculated using the method of Karber (G. Karber, *Arch Exp. Path. Pharmakol.*, 162:480, 1931). L235 (○), L238 (△), M118 (■), L652 (●), and L471 (□). These titers rose rapidly and peaked at 5 months postinfection and remained constant throughout the following year. Neutralization assays were routinely done in a T-cell line (CEMx174), however, when the assays were done in primary rhesus macrophages, an equivalent level of neutralizing antibody was measured. Neutralization assays were done in primary macaque macrophages as described in FIGURE 2 except that primary macaque macrophages were cultured in 96 well plates for 5 days prior to the addition of virus or virus incubated with serial dilutions of plasma. The

endpoint was determined at fourteen days by the addition of 2×10^6 CEMx174 cells/well, the CPE assessed, and the 50% neutralization endpoint determined. The production of high levels of neutralizing antibodies in monkeys infected with SIV/17E-Cl is in direct contrast to the absence or low levels of neutralizing antibodies made in response to infection with the parental strain $SIV_{mac}239$. Thus, envelope sequences in SIV/17E-Cl that confer macrophage-tropism also appear to be responsible for eliciting a strong neutralizing antibody response *in vivo*.

To determine the specificity of the neutralizing antibodies, neutralizations were done at monthly intervals using $SIV_{mac}239$, the uncloned parental strain SIV/17E-Br, another recombinant clone that contains the entire *env* gene from SIV_{mac}/17E-Cl called SIV_{mac} 17E-Fr, and a heterologous primary isolate of sooty mangabey monkey origin, SIV/DeltaB670 (Table 3).

TABLE 3

SIV/DeltaB670-SPECIFIC NEUTRALIZATION TITER*

Monkey	DL235	L238	L471	L652	M118
90	n.t.	0	n.t.	1.25	n.t.
118	n.t.	0	0	1.25	0
150	0	0	0	1.0	0
195	0.7	0	0.7	1.75	0
219	1.0	1.0	1.0	1.75	1.2
244	1.0	1.0	n.t.	n.t.	n.t.

* 50% neutralization endpoint, log 10
n.t. = not tested.

Within the first 150 days postinoculation, with the exception of monkey L652, sera obtained from the SIV/17E-Cl infected monkeys neutralized only SIV/17E-Cl. In contrast, samples obtained later neutralized not only SIV/17E-

Cl, but also SIV/17E-Br, the recombinant virus SIV/17E-Fr, and the heterologous isolate, SIV/DeltaB670 (Table 4).

TABLE 4

NEUTRALIZATION TITER*

Monkey	SIV/17E-Cl	SIV/17E-Br	SIV/17-Fr	SIV/DeltaB-670
L235	4.3	0.7	0.7	1.0
L238	4.8	0	2.3	1.0
L471	4.5	0	2.3	1.0
L652	3.4	1.8	3.0	1.75
M118	4.5	0.9	2.6	1.2
C344+	4.2	n.t.	n.t.	2.1

*50% Neutralization endpoint, log 10 of sera taken 219 days postinoculation.
+Long-term survivor of SIV/DeltaB670 infection
n.t.= not tested.

The neutralizing titer against the heterologous primary isolate, SIV/DeltaB670, never achieved the titers observed with the homologous clone, SIV/17E-Cl. Indeed, the neutralizing titers observed in monkey C344, a long-term survivor of SIV/DeltaB670 infection (1 of only 2 survivors of over 400 animals inoculated with this virus), were 2 logs less than that for SIV/17E-Cl (Table 4). Nevertheless, the pattern of neutralizing activity was highly consistent in multiple samples taken throughout the infection in all the animals tests, and with respect to repeated determinations performed on the same sample. The rapid appearance of broadly-reactive neutralizing activity in monkey L652 remains unexplained at present. However, for the majority of these animals, a broadening of neutralizing antibody from type-specific to group-specific activity was observed.

The ability of antisera raised against SIV/17E-Cl late in the infection to neutralize SIV/DeltaB670 was surprising given the genetic composition of the two viruses. Unlike SIV/17E-Cl which consists of a single genotype of

SIV_{mac}251 lineage, SIV/DeltaB670 is a primary isolate consisting of a swarm of genetic variants cultured from the lymph node of a rhesus monkey infected with SIV from a sooty mangabey monkey. Sequence comparison of the V1 hypervariable region of *env* found within the SIV/DeltaB670 swarm identified a divergence of less than 10%, whereas a similar comparison of these sequences to SIV/17E-Cl showed an average divergence of over 25%. To place this difference in perspective, a comparison of SIV.17E-Cl V1 sequences to those obtained from the Los Alamos database for SIV_{mac}251, SIV_{mne}, SIV/STM, and HIV-2ROD showed divergences of 9.5%, 16%, 30%, and 40%, respectively. Thus, persistent infection with the attenuated molecular clone SIV/17E-Cl induces, over time, neutralizing antibodies against a genetically diverse strain of SIV.

EXAMPLE 7
AVIDITY AND CONFORMATION OF ANTIBODY RESPONSES TO SIV/17E-Cl

In light of the rapid appearance of type-specific neutralizing responses and the delayed appearance of neutralizing antibody responses with broader specificity in macaques infected with SIV/17E-Cl, the evolution of SIV envelope glycoprotein-specific antibody responses with respect to their activity and conformational dependence was examined. For these purposes, native viral glycoproteins from purified virus preparations were anchored onto concanavalin A in microtiter plates for the respective immunoassays. Serum antibody avidities were determined by measuring the stability of antibody-antigen complexes to a urea wash as described previously (K. Hedman and S.A. Rousseau, *J. Med. Virol.*, 27:288, 1989; K. Hedman, *et al., J. Med. Virol.*, 27:293, 1989). In this functional assay, avidity index values below 30% are considered low avidity antibody, values between 30% and 50% are considered intermediate avidity, and values above 50% are designated as high avidity antibodies.

FIGURE 7 shows avidity of serum antibodies for SIV envelope glycoproteins. The antibody avidity index (AI) (K. Hedman, *et al., supra*) was determined by measuring in Con A-ELISA (J.E. Robinson, *et al., J. Immunol. Meth.*, 132:63, 1990) the resistance of test serum antibody-envelope glycoprotein immune complexes to disruption by treatment with 8 M urea. Longitudinal serum samples were obtained from five macaques at various times after infection with SIV/17E-Cl: L235 (○), L238 (△), M118 (■), L652 (●), and L471 (□). Con A-anchored native envelope glycoprotein substrate was prepared from Triton X-100-disrupted SIVB7 in microtiter plates as described in FIGURE 4. SIVB7 is a noninfectious virus derived from CEMx174 cells chronically infected with $SIV_{mm}H3$ generously provided by Edmundo Kraiselburd at the Primate Center at the University of Puerto Rico. Test sera, diluted to give an absorbency at 450 nm of about 1.0, were reacted with the Con A-glycoprotein substrate and the wells washed with PBS-TX. Triplicate wells containing the glycoprotein immune complexes were then treated in parallel for 5 minutes with either PBS or a solution 8 M urea in PBS. Following this treatment, sample wells were washed thoroughly with PBS-TX, incubated with TMBlue, and the color development measured at 450 nm. The avidity index (AI) was calculated from the ratio (A/B x 100%) of the absorbency values obtained with the urea treatment FIGURE 7(A) compared to the absorbency observed with the PBS treatment, FIGURE 7(B). AI values ⟨30% are designated as low avidity antibodies, 30%-50% as intermediate avidity antibodies, and ⟩50% as high avidity antibodies (K. Hedman, *et al., supra*).

The data in FIGURE 7 demonstrate that the envelope glycoprotein-specific antibody responses in the 5 SIV/17E-Cl infected monkeys evaluated longitudinally increase in avidity over the first 7 months post infection and apparently level off at an intermediate avidity of about 40% thereafter. The relatively slow evolution of antibody avidity in the glycoprotein-specific antibody response indicates an ongoing maturation of humoral immune responses to this chronic infection for at least 7 months postinfection. At this time, the avidity appears to reach a maximum level that is maintained even after subsequent virus

challenge 8 months postinfection. This slow increase in the avidity of antibodies to the SIV envelope glycoprotein is in distinct contrast to the relatively rapid increase in avidities observed during other viral infections (A. Salmi, *Curr. Opin. Immunol.*, 3:503, 1992), perhaps indicating an important escape mechanism by which SIV eludes immune responses soon after infection.

The conformation-dependence of envelope-specific antibody responses was also measured in the longitudinal panel of serum samples taken from 5 of the 8 SIV/17E-Cl-infected monkeys. For this assay, the relative reactivity of serum antibodies at a standard dilution was measured in parallel against con A-anchored native viral glycoprotein and against "denatured" viral glycoprotein produced by reductive carboxymethylation of protein cysteine sulfhydryl groups. Thus, this assay compares the reactivity of serum antibodies with a native viral glycoprotein complex to that with envelope glycoproteins in which all disulfide bonds have been irreversibly reduced to alter protein tertiary structure, without deliberate denaturation of the envelope protein secondary structure.

FIGURE 8 shows conformational dependence of serum antibodies to SIV envelope glycoproteins. The conformational dependence of envelope-specific antibodies elicited by infection with SIV/17D-Cl was determined by measuring in Con A-ELISA (J.E. Robinson, *et al., J. Immunol. Meth.*, 132:63, 1990) the antibody reactivities against a native (panel A) and denatured (panel B) viral envelope glycoprotein substrates prepared by reduction and carboxymethylation of protein sulfhydryl groups. For these assays, gradient-purified (M. Murphey-Corb, *et al., supra*) SIVB7 particles were used. Purified SIVB7 particles disrupted with 1% Triton X-100 was used as the "native" glycoprotein substrate. To produce the "denatured" glycoprotein substrate, SIVB7 was treated with dithiothreotol to reduce disulfide bonds and then with iodoacetic acid to achieve an irreversible carboxymethylation of the reduced sulfhydryl groups (A.M. Crestfield, *et al., J. Biol. Chem.*, 38:622, 1963). These

reaction conditions were chosen because they should quantitatively disrupt envelope glycoprotein disulfide bonds and affect tertiary protein structure without extensive alterations in protein secondary structural properties, as would be expected from treatments with chaotropic salts or ionic detergents.

Immunolon II Microtiter plates (Dynatech Lab., Chantilly, VA) were incubated with 2.5 μl Con A (Vector Laboratories, Burlingame, CA) per well in 50 μl PBS, pH 7.4, for 1 hour at room temperature and then washed 2 times with PBS containing 0.1% Triton X-100 (PBS-TX). The Con A-coated plates were then used to adsorb respective preparations of gp120 (3 μg/50 μl in each well). Con A-adsorbed gp120 was washed 4 times with PBS-TX and blocked with 100 μl per well of 5% nonfat dry mild in PBS (blotto) for 1 hour at room temperature. After removing the blocking solution, 50 μl of appropriately diluted test serum were added to each well and incubated for 1 hour at room temperature. All test sera were diluted in blotto to produce an absorbency at 450 nm of about 1.0 in the standard Con A-ELISA procedure. After serum incubation, the wells were washed with PBS-TX, and 50 μl per well of a 1:1000 dilution of peroxidase-conjugated anti-human IgG in blotto was added for 1 hour at room temperature. The wells were once again washed with PBS-TX and 200 μl of TMBlue (TSI) substrate was added for approximately 15 minutes before color development was terminated by the addition of 50 μl per well of 1 N sulfuric acid. Antibody reactivity to the Con A-anchored native or denatured envelope glycoprotein substrates was then determined by measuring the absorbency at 450 nm. (Monkeys L235 (○), L238 (△), M118 (■), L652 (●), and L471 (□)).

Serum antibodies at all time points tested displayed a 2-3 fold greater reactivity with the native viral glycoprotein substrate compared to the denatured viral glycoprotein antigen (FIGURE 8). The predominance of conformation dependent antibodies consistently identified in sera of SIV/17E-Cl-infected monkeys is reminiscent of similar antibodies produced in chronic HIV-1 (J.P. Moore and D.D. Ho, *Virol.*, 67:863, 1993) and SIV (B.W. McBride,

et al., Gen. Virol., 74:1033, 1993) infections. This is the first report of the in kinetics of induction of this conformational dependent response over the course of an experimental infection, thereby revealing the early dominance of this response. In this regard, the conformational dependence properties of the antibody response to SIV/17E-C1 infection differ from the avidity properties which clearly evolve more slowly over the first 7 months postinfection.

EXAMPLE 8
LACK OF HETEROGENEITY OF ENV SEQUENCES IN MACAQUES INFECTED WITH SIV/17E-CI

To examine if the broadening of the neutralizing antibody response and increased avidity of the antibody for the viral glycoprotein was due to the development of heterogeneity in *env* protein composition, the genetic diversity of gp120 sequences in virus present in two of the animals that were challenged (L235 and L238) was examined nine days after inoculation in the peripheral blood mononuclear cells and at 244 days after inoculation in mononuclear cells from both lymph nodes and the peripheral blood. The entire gp120 sequence 3 (nucleotides 6342-8222; (D.A. Regier and R.C. Desrosiers, *AIDS Res. Hum. Retroviruses*, 6:1221 1990). For sequence analysis amplification of the entire gp120 envelope sequence was performed by nested PCR using the conditions described in EXAMPLE 5 and primers (5'): 5'-TTGAGGGAGCAGGAGAACTCATTA-3' (SEQ ID NO:22) and (3') 5'-CCAGGCGGCGACTAGGAGAGATGGGAACAA-3' (SEQ ID NO:23) for the first round and (5'): 5'-CCTCCAACGAGCGCTCTTCAT-3' (SEQ ID NO:24), and (3'): 5'-CCTGCTGTTGCGAGAAAACCCAAGAACCCTAGC-3' (SEQ ID NO. 25) for the second round. Sequences specific for the external domain of gp41 were similarly amplified using primers (5') 5'-GAACATACATTT-ATTGGCATCCTAG-3' (SEQ ID NO:26) and (3') 5'-AAGCAGAAAGGGTCCTAACAGACCAGGGT-3' (SEQ. ID NO:27) for the first round and (5') 5'-CCATTGGTCAAACATCCCACATATACTGGA-3' (SEQ ID

NO:28) and (3') 5'-CCAGGCGGCGACTAGGAGAGATGGGAACAG-3' (SEQ ID NO:29) for the second round. Sequences specific for the external domain of gp41 were similarly amplified using primers (5') 5'-GAACATACATTTATTGGCATCCTAG-3' (SEQ ID NO:30) and (3') 5'-AAGCAGAAAGGGTCCTAACAGACCAGGGT-3' (SEQ ID NO:31) for the first round and (5') 5'-CCATTGGTCAAACATCCCACATATACTGGA-3' (SEQ ID NO:32) and (3') 5'-CCAGGCGGCGACTAGGAGAGATGGGAACAG-3' (SEQ ID NO:33) for the second round. Second round products were cloned into the TA cloning vector (Invitrogen, San Diego, CA). Following transformation, colonies containing appropriately sized inserts were selected, and plasmid DNA purified by alkaline lysis. Inserts containing the envelope region was sequenced by dideoxy sequencing using Sequenase (U.S. Biochemical, Cleveland, OH). The resulting sequences were analyzed using the "dots" alignment program provided by the Mullins laboratory at Stanford University assisted by a SUN sparkstation).

Very few nucleotide changes were detected in all clones examined. A comparison of early and late samples failed to reveal any significant difference in either the number of mutations or location of these changes. This low level of virus heterogeneity is in direct contrast to infection with the lymphocyte-tropic molecular clone $SIV_{mac}239$ which rapidly generates genetic diversity within this same time frame (D.P.W. Burnes and R.C. Desrosiers, *J. Virol.*, 65:1843, 1991; D.P.W. Burnes, *et al., J. Virol.*, 67:4104, 1993) a factor which is probably due to the low level of replication observed in the SIV/17E-Cl-infected animals. These data suggest that development of cross-reactive neutralizing antibodies was likely not due to the generation and selection of antigenic variants or to the presence of a widely divergent virus swarm in these monkeys, but rather may reflect the recognition of less immunogenic viral epitopes by the immune system as these responses mature.

EXAMPLE 9

CHALLENGE OF SIV/17E-CI-INFECTED MACAQUES WITH A HETEROLOGOUS STRAIN OF SIV

To determine whether the broadened neutralization response that appeared after 7 months postinfection in the SIV/17E-Cl infected macaques could confer broad-spectrum protective immunity against heterologous virus challenge, infected macaques were inoculated intravenously with 50 animal infectious doses of rhesus-grown SIV/DeltaB670 either prior to 7 months (3 macaques) or after 8 months (2 macaques) postinfection (TABLE 4). Lymph node biopsies were performed immediately prior to and 14 days after challenge and, together with PBMC-derived cells, were evaluated for SIV/DeltaB670-specific sequences by PCR. SIV-specific sequences, when present, were further analyzed by sequence analysis of cloned PCR products containing the V1 hypervariable domain of gp120 (Sequences specific for the V1 hypervariable region of gp120 were determined on PCR-amplified products as described above using primers (5') 5'-CCTCCAACGAGCGCTCTTCAT-3' (SEQ ID NO:34) and (3') 5'-CCTGCTGTTGCGAGAAAACCCAAG-AACCCTAGC-3' (SEQ ID NO:35) for the first round and (5') 5'-CAGTCACAGAACAGGCAATAGA-3' (SEQ ID NO:36) and (3') 5'-CCTGCTGTTGCGAGAAAACCCAAGAACCCTAGC-3' (SEQ ID NO:37) for the second round.

No evidence of infection with SIV/DeltaB670 could be identified in either of the monkeys infected for more than 8 months which had broadly reactive neutralizing responses (L235, L238). In contrast, all three macaques (M697, M462, M700) challenged earlier in infection had SIV/DeltaB670-specific sequences in both PBMC's and lymph node derived mononuclear cells; only one of these animals (monkey M697) had detectable neutralizing antibody to SIV/DeltaB670 at challenge. Multiple SIV/DeltaB670 variants found within the challenge inoculum were identified in monkey M700; this animal died of AIDS-

like disease 95 days postchallenge. The major variant found within the challenge inoculum was identified in monkey M462, whereas only a minor variant (representing less than 10% of the variants) could be identified in monkey M697. Similar analysis of naive monkeys inoculated with SIV/DeltaB670 has shown that the major variant present in the initial inoculum consistently emerges as the dominant form following *in vivo* infection. Taken together, these data suggest that the clonal emergence of a minor form of the inoculum observed in monkey M697 may have been influenced by the low level of neutralizing antibody detected in the serum of this animal at challenge. Both M462 and M697 have lived beyond 280 days postchallenge, but are showing persistent declines in CD4+ and increases in CD8+ T lymphocytes in the peripheral blood.

Infection of rhesus macaques with an attenuated macrophage-tropic clone of SIV induced a response which protected against challenge with a highly virulent primary isolate that differs by 16% overall in gp120 nucleotide sequence and 25% in the sequence of the V1 hypervariable region of gp120. The induction of this protective response occurred concomitantly with the broadening of neutralizing antibody responses apparent after 7 months postinfection.

Evaluation of neutralizing antibody induced by the attenuated clone demonstrated the early (within 2 weeks) induction of a vigorous response specific to the infecting clone which broadened by 7 months to include neutralizing activity against the heterologous challenge isolate. Glycoprotein-specific antibody responses were mainly directed toward conformation dependent epitopes, with the avidity of these responses requiring 7 months to mature. Comparative analysis of gp120 sequences obtained early in infection to those acquired at challenge revealed no apparent sequence diversity to account for the changes observed, suggesting that more subtle changes, perhaps in immunological maturation, may be involved. These findings, coupled with the failure to demonstrate any evidence of viral infection or replication either by

PCR-assisted sequence analysis or by the identification of an amnestic response early after challenge suggests that sterilizing immunity was achieved.

EXAMPLE 10

FURTHER CHARACTERIZATION OF THE ROLE OF ANTIBODY RESPONSES IN THE PROTECTION OBSERVED FOR SIV/17E-CL INFECTION

The inability to detect sequences specific for the challenge virus within the first 2 weeks postchallenge of monkeys infected with the SIV/17E-Cl is consistent with the induction of sterilizing immunity. To confirm this hypothesis, sera was collected from the two protected monkeys, pooled, heat inactivated, and given to 4 naive recipients at a concentration of 10 ml/kg per monkey (see below). Two control monkeys received the same volume of sera from uninfected macaques. Seven hours later, these animals were challenged intravenously with 50 animal infectious doses of rhesus-grown SIV/DeltaB670. The two control monkeys were both PCR positive at 7 days postchallenge. Of the monkeys receiving immune sera, one monkey (N549) was PCR positive at 7 days; another became PCR positive at 14 days (M954) postchallenge. The remaining two monkeys have remained virus negative for more than 6 months postchallenge.

Although the differences in antibody titer versus protection were not statistically significant, the monkey receiving immune sera that was clearly infected by 7 days postchallenge had the lowest titer, a finding that is consistent with protection induced by antibody to the virus. Thus, monocytotropic SIV/17E-Cl serves as an effective vaccine by inducing protective responses that are, at least in part, humoral.

TABLE 5

MONKEY	gp140 titer*	PCR results P.C.+
N261	1600	-
N549	800	+
N644	1600	-
M954	1600	+ (@ 14 days)
Donor serum	6400	

* Reciprocal dilution

+ P.C. = postchallenge

EXAMPLE 11

IMMUNOLOGICAL COMPARISONS BETWEEN SIV/17EΔnef VERSUS SIV239Δnef INFECTION IN THE RHESUS MONKEY

To determine whether the immune responses induced by SIV/17E-Cl (monocyte-tropic) were indeed qualitatively different from those induced by SIVmac239Δnef (lymphocyte-tropic) infection, two congenic clones, SIVmac17EΔnef and SIVmac239Δnef, were constructed using the same deletion strategy originally described by Dr. Ron Desrosiers for SIVmac239Δnef (Desrosiers, *et al., supra.*) Six monkeys were inoculated with each strain and monitored for infection, disease status, and immune responses to viral infection. All monkeys became persistently infected and have shown no signs of disease at 8 months postinfection.

MHC class I restricted CTL assays were performed at 6 and 8 months postinfection (FIGURE 9). Three of 4 monkeys tested at 6 months and 2 of 6 monkeys tested at 8 months postinfection with SIV/17EΔnef had detectable CTL responses to env, gag, and pol proteins. In contrast, none of 4 tested at 6 months, and none of 6 monkeys tested at 8 months postinfection with SIV239Δnef had detectable CTL. Interestingly, the gp140-specific antibody titers in SIV/17EΔnef-infected animals correlated inversely with the detection

of CTL activity, in a manner obtained by DNA immunizations. Moreover, as expected, neutralizing antibody responses were identified only in those monkeys infected with SIV/17EΔnef.

These results show clear differences in the immune response elicited by replication of the two congenic clones, and reinforces the concept that monocyte-tropic variants may offer a selective advantage lymphocyte-tropic sequences in the induction of immune responses that are protective.

The early (within 3 weeks) induction of vigorous type-specific neutralizing antibody responses in SIV/17E-Cl infected monkeys is in dramatic contrast to the absence of detectable neutralizing antibody in monkeys infected with the parent clone $SIV_{mac}239$ where weak responses only appear after 6 months postinfection. The genetic difference between $SIV_{mac}239$ and SIV/17E-Cl is restricted to 7 amino acid changes in gp160; these conservative changes not only confer the ability to replicate in monocyte/macrophages but also enable the early induction of vigorous neutralizing activity *in vivo*. These changes may promote conformational changes in the envelope which expose neutralizing epitopes, or neutralizing antibody may be more readily induced by presentation to the immune system by infected monocyte/macrophages, or both.

This is the first report describing the induction of heterologous protection by an attenuated macrophage-tropic clone of SIV, and characterization of the specific responses associated with this protection. Similar conformation-dependent neutralizing antibodies with a broad specificity against primary isolates have been described in chronically-infected humans (K.S. Steimer, *et al., Science*, 254:105, 1991; J.P. Moore, *et al., supra*) and monkeys (B.W. McBride, *et al., Gen. Virol.*, 74:1033, 1993).

The data reported here suggest that an HIV vaccine must fulfill two requirements for the induction of protective immunity: 1) presentation of confor-

mationally correct proteins, and 2) induction of the appropriate responses. For the development of safe subunit vaccines, newer methodologies using immunization with DNA (Braciale, T.J., *Trends in Microbiology*, 1:P323, 1993; Ulmer, *et al., Science*, 259:1745, 1993) and live virus vectors are likely candidates for fulfilling the first requirement. Induction of the appropriate response, however, may be more difficult to achieve since, in infection with attenuated strains, these responses are induced slowly over the course of continued immune stimulation. However, the use of specific adjuvants formulated to selectively drive these responses, once identified, may accomplish this task.

The utilization of viral proteins conferring macrophage-tropism may offer a selective advantage over the lymphocyte-tropic analogs that have dominated vaccine development thus far. Macrophage-tropic strains of HIV-1 appear to be transmitted most efficiently and to establish initial infections in humans (T.F.W. Wolfs, *et al., Virol.*, 189:103, 1992; S.M. Wolinsky, *et al., Science*, 255:1134, 1992; L.Q. Zhang, *et al., J. Virol.*, 67:3345, 1993; T. Zhu, *et al., Science*, 261:1179, 1993) and are therefore the initial targets for vaccine-induced protection. These strains may also be better inducers of protective responses. The vigorous early induction of neutralizing antibody responses observed in SIV/17E-Cl-infected monkeys is in striking contrast to the absence of these responses in monkeys infected with SIVmac239, which differs by only 7 amino acid residues in gp120 (M.G. Anderson, *et al., Virol.*, 195:616, 1993). Comparative analysis of immune responses induced by the two infectious clones should identify conformational differences which may be critical to the ongoing development of an effective vaccine for HIV.

The foregoing is meant to illustrate, but not to limit, the scope of the invention. Indeed, those of ordinary skill in the art can readily envision and produce further embodiments, based on the teachings herein, without undue experimentation.

SEQUENCE LISTING

(1) GENERAL INFORMATION

(i) APPLICANT: The Johns Hopkins University School of Medicine

(ii) TITLE OF THE INVENTION: METHOD OF TREATMENT OF HUMAN IMMUNODEFICIENCY VIRUS (HIV) INFECTION

(iii) NUMBER OF SEQUENCES: 38

(iv) CORRESPONDENCE ADDRESS:
(A) ADDRESSEE: Fish & Richardson P.C.
(B) STREET: 4225 Executive Square, Suite 1400
(C) CITY:La Jolla
(D) STATE: CA
(E) COUNTRY: USA
(F) ZIP: 92037

(v) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Diskette
(B) COMPUTER: IBM Compatible
(C) OPERATING SYSTEM: DOS
(D) SOFTWARE: FastSEQ Version 1.5

(vi) CURRENT APPLICATION DATA:
(A) APPLICATION NUMBER: PCT/US95/
(B) FILING DATE: 19-SEP-1995
(C) CLASSIFICATION:

(vii) PRIOR APPLICATION DATA:
(A) APPLICATION NUMBER:
(B) FILING DATE:

(viii) ATTORNEY/AGENT INFORMATION:
(A) NAME: Haile, Ph.D., Lisa A.
(B) REGISTRATION NUMBER: 38,347
(C) REFERENCE/DOCKET NUMBER: 07265/038WO1

(ix) TELECOMMUNICATION INFORMATION:
(A) TELEPHONE: 619-678-5070
(B) TELEFAX: 619-678-5099
(C) TELEX:

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 36 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA
(iii) HYPOTHETICAL: NO
(iv) ANTISENSE: NO
(v) FRAGMENT TYPE:
(vi) ORIGINAL SOURCE:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

AAGCTTGGAT CCGCATGCTA TAACACATGC TATTGT 36

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 36 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA
(iii) HYPOTHETICAL: NO
(iv) ANTISENSE: NO
(v) FRAGMENT TYPE:
(vi) ORIGINAL SOURCE:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

AAGCTTGAAT TCGGAGGTTC TTTGTTCCCC AGACGG 36

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 23 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA
(iii) HYPOTHETICAL: NO
(iv) ANTISENSE: NO
(v) FRAGMENT TYPE:

(vi) ORIGINAL SOURCE:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

GCTTTGCTTA GATGTAATGA CAC 23

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA
(iii) HYPOTHETICAL: NO
(iv) ANTISENSE: NO
(v) FRAGMENT TYPE:
(vi) ORIGINAL SOURCE:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

TGGATATGGG TCTGCTGGAA 20

(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 36 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA
(iii) HYPOTHETICAL: NO
(iv) ANTISENSE: NO
(v) FRAGMENT TYPE:
(vi) ORIGINAL SOURCE:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

AAGCTTGGAT CCGGCTTGGG GATATGTTAT GAGCAA 36

(2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 22 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA
(iii) HYPOTHETICAL: NO
(iv) ANTISENSE: NO
(v) FRAGMENT TYPE:
(vi) ORIGINAL SOURCE:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

CCTGGTCTTC TACATTTCAT TG 22

(2) INFORMATION FOR SEQ ID NO:7:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 21 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA
(iii) HYPOTHETICAL: NO
(iv) ANTISENSE: NO
(v) FRAGMENT TYPE:
(vi) ORIGINAL SOURCE:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

TGCACAAGGA TGATGGAGAC A 21

(2) INFORMATION FOR SEQ ID NO:8:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 22 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA
(iii) HYPOTHETICAL: NO
(iv) ANTISENSE: NO

(v) FRAGMENT TYPE:
(vi) ORIGINAL SOURCE:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

CTCTATCGAT TGGAATTGGG AG 22

(2) INFORMATION FOR SEQ ID NO:9:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 32 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA
(iii) HYPOTHETICAL: NO
(iv) ANTISENSE: NO
(v) FRAGMENT TYPE:
(vi) ORIGINAL SOURCE:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

AAGCTTGGAT CCCTCCAACG AGCGCTTTTA AT 32

(2) INFORMATION FOR SEQ ID NO:10:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 33 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA
(iii) HYPOTHETICAL: NO
(iv) ANTISENSE: NO
(v) FRAGMENT TYPE:
(vi) ORIGINAL SOURCE:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

AAGCTTGGAT CCCCCCTGCC TTAACTTAGC TAG 33

(2) INFORMATION FOR SEQ ID NO:11:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 36 base pairs
(B) TYPE: nucleic acid

(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA
(iii) HYPOTHETICAL: NO
(iv) ANTISENSE: NO
(v) FRAGMENT TYPE:
(vi) ORIGINAL SOURCE:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

AAGCTTCTCG AGCATTTGGC TGGCTATGGA AATTAG 36

(2) INFORMATION FOR SEQ ID NO:12:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 29 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA
(iii) HYPOTHETICAL: NO
(iv) ANTISENSE: NO
(v) FRAGMENT TYPE:
(vi) ORIGINAL SOURCE:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:

AAGTTGGATC CCTCTACCTG CTAGTGCTG 29

(2) INFORMATION FOR SEQ ID NO:13:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 34 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA
(iii) HYPOTHETICAL: NO
(iv) ANTISENSE: NO
(v) FRAGMENT TYPE:
(vi) ORIGINAL SOURCE:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:13:

AAGCTTGAAT TCGCATCAGC AAAAGTAGAC ATGG 34

(2) INFORMATION FOR SEQ ID NO:14:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 23 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA
(iii) HYPOTHETICAL: NO
(iv) ANTISENSE: NO
(v) FRAGMENT TYPE:
(vi) ORIGINAL SOURCE:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:14:

CTCTTGACCA CATCCAACAG CTG 23

(2) INFORMATION FOR SEQ ID NO:15:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 23 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA
(iii) HYPOTHETICAL: NO
(iv) ANTISENSE: NO
(v) FRAGMENT TYPE:
(vi) ORIGINAL SOURCE:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:15:

CAGCTGTTGG ATGTGGTCAA GAG 23

(2) INFORMATION FOR SEQ ID NO:16:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 33 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA
(iii) HYPOTHETICAL: NO
(iv) ANTISENSE: NO
(v) FRAGMENT TYPE:
(vi) ORIGINAL SOURCE:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:16:

AAGCTTGGAT CCCCCCTGCC TTAACTTAGC TAG 33

(2) INFORMATION FOR SEQ ID NO:17:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 24 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA
(iii) HYPOTHETICAL: NO
(iv) ANTISENSE: NO
(v) FRAGMENT TYPE:
(vi) ORIGINAL SOURCE:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:17:

ATAGTTGCAG TACATGTGGC TAGT 24

(2) INFORMATION FOR SEQ ID NO:18:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 26 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA
(iii) HYPOTHETICAL: NO
(iv) ANTISENSE: NO
(v) FRAGMENT TYPE:
(vi) ORIGINAL SOURCE:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:18:

TCTCTGCCTC TTTCTCTGTA ATAGAC 26

(2) INFORMATION FOR SEQ ID NO:19:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 28 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA
(iii) HYPOTHETICAL: NO
(iv) ANTISENSE: NO
(v) FRAGMENT TYPE:
(vi) ORIGINAL SOURCE:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:19:

AGGCAGAAAG GGTCCTACAG ACCAGGGT 28

(2) INFORMATION FOR SEQ ID NO:20:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 28 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA
(iii) HYPOTHETICAL: NO
(iv) ANTISENSE: NO
(v) FRAGMENT TYPE:
(vi) ORIGINAL SOURCE:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:20:

AGGCAGAAAG GGTCCTACAG ACCAGGGT 28

(2) INFORMATION FOR SEQ ID NO:21:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 22 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA
(iii) HYPOTHETICAL: NO
(iv) ANTISENSE: NO
(v) FRAGMENT TYPE:
(vi) ORIGINAL SOURCE:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:21:

AGCAGGTAGA GCCTGGGTGT TC 22

(2) INFORMATION FOR SEQ ID NO:22:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 24 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA
(iii) HYPOTHETICAL: NO
(iv) ANTISENSE: NO
(v) FRAGMENT TYPE:
(vi) ORIGINAL SOURCE:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:22:

TTGAGGGAGC AGGAGAACTC ATTA 24

(2) INFORMATION FOR SEQ ID NO:23:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 30 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA
(iii) HYPOTHETICAL: NO
(iv) ANTISENSE: NO
(v) FRAGMENT TYPE:
(vi) ORIGINAL SOURCE:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:23:

CCAGGCGGCG ACTAGGAGAG ATGGGAACAA 30

(2) INFORMATION FOR SEQ ID NO:24:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 21 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA
(iii) HYPOTHETICAL: NO
(iv) ANTISENSE: NO
(v) FRAGMENT TYPE:
(vi) ORIGINAL SOURCE:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:24:

CCTCCAACGA GCGCTCTTCA T 21

(2) INFORMATION FOR SEQ ID NO:25:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 33 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA
(iii) HYPOTHETICAL: NO
(iv) ANTISENSE: NO
(v) FRAGMENT TYPE:
(vi) ORIGINAL SOURCE:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:25:

CCTGCTGTTG CGAGAAAACC CAAGAACCCT AGC 33

(2) INFORMATION FOR SEQ ID NO:26:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 25 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA
(iii) HYPOTHETICAL: NO
(iv) ANTISENSE: NO
(v) FRAGMENT TYPE:
(vi) ORIGINAL SOURCE:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:26:

GAACATACAT TTATTGGCAT CCTAG 25

(2) INFORMATION FOR SEQ ID NO:27:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 29 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA
(iii) HYPOTHETICAL: NO
(iv) ANTISENSE: NO
(v) FRAGMENT TYPE:
(vi) ORIGINAL SOURCE:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:27:

AAGCAGAAAG GGTCCTAACA GACCAGGGT 29

(2) INFORMATION FOR SEQ ID NO:28:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 30 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA
(iii) HYPOTHETICAL: NO
(iv) ANTISENSE: NO
(v) FRAGMENT TYPE:
(vi) ORIGINAL SOURCE:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:28:

CCATTGGTCA AACATCCCAC ATATACTGGA 30

(2) INFORMATION FOR SEQ ID NO:29:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 30 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA
(iii) HYPOTHETICAL: NO
(iv) ANTISENSE: NO
(v) FRAGMENT TYPE:

(vi) ORIGINAL SOURCE:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:29:

CCAGGCGGCG ACTAGGAGAG ATGGGAACAG 30

(2) INFORMATION FOR SEQ ID NO:30:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 25 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA
(iii) HYPOTHETICAL: NO
(iv) ANTISENSE: NO
(v) FRAGMENT TYPE:
(vi) ORIGINAL SOURCE:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:30:

GAACATACAT TTATTGGCAT CCTAG 25

(2) INFORMATION FOR SEQ ID NO:31:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 29 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA
(iii) HYPOTHETICAL: NO
(iv) ANTISENSE: NO
(v) FRAGMENT TYPE:
(vi) ORIGINAL SOURCE:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:31:

AAGCAGAAAG GGTCCTAACA GACCAGGGT 29

(2) INFORMATION FOR SEQ ID NO:32:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 30 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA
(iii) HYPOTHETICAL: NO
(iv) ANTISENSE: NO
(v) FRAGMENT TYPE:
(vi) ORIGINAL SOURCE:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:32:

CCATTGGTCA AACATCCCAC ATATACTGGA 30

(2) INFORMATION FOR SEQ ID NO:33:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 30 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA
(iii) HYPOTHETICAL: NO
(iv) ANTISENSE: NO
(v) FRAGMENT TYPE:
(vi) ORIGINAL SOURCE:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:33:

CCAGGCGGCG ACTAGGAGAG ATGGGAACAG 30

(2) INFORMATION FOR SEQ ID NO:34:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 21 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA
(iii) HYPOTHETICAL: NO
(iv) ANTISENSE: NO
(v) FRAGMENT TYPE:
(vi) ORIGINAL SOURCE:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:34:

CCTCCAACGA GCGCTCTTCA T 21

(2) INFORMATION FOR SEQ ID NO:35:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 33 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA
(iii) HYPOTHETICAL: NO
(iv) ANTISENSE: NO
(v) FRAGMENT TYPE:
(vi) ORIGINAL SOURCE:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:35:

CCTGCTGTTG CGAGAAAACC CAAGAACCCT AGC 33

(2) INFORMATION FOR SEQ ID NO:36:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 22 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA
(iii) HYPOTHETICAL: NO
(iv) ANTISENSE: NO
(v) FRAGMENT TYPE:
(vi) ORIGINAL SOURCE:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:36:

CAGTCACAGA ACAGGCAATA GA 22

(2) INFORMATION FOR SEQ ID NO:37:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 33 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA
(iii) HYPOTHETICAL: NO
(iv) ANTISENSE: NO
(v) FRAGMENT TYPE:

(vi) ORIGINAL SOURCE:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:37:

CCTGCTGTTG CGAGAAAACC CAAGAACCCT AGC 33

(2) INFORMATION FOR SEQ ID NO:38:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 731 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide
(iii) HYPOTHETICAL: NO
(iv) ANTISENSE: NO
(v) FRAGMENT TYPE: N-terminal
(vi) ORIGINAL SOURCE:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:38:

```
Met Gly Cys Leu Gly Asn Gln Leu Leu Ile Ala Ile Leu Leu Leu Ser
 1               5                  10                  15
Val Tyr Gly Ile Tyr Cys Thr Leu Tyr Val Thr Val Phe Tyr Gly Val
            20                  25                  30
Pro Ala Trp Arg Asn Ala Thr Ile Pro Leu Phe Cys Ala Thr Asn Lys
        35                  40                  45
Arg Asp Thr Trp Gly Thr Thr Gln Cys Leu Pro Asp Asn Gly Asp Tyr
    50                  55                  60
Ser Glu Val Ala Leu Asn Val Thr Glu Ser Phe Asp Ala Trp Asn Asn
65                  70                  75                  80
Thr Val Thr Glu Gln Ala Ile Glu Asp Val Trp Gln Leu Phe Glu Thr
                85                  90                  95
Ser Ile Lys Pro Cys Val Lys Leu Ser Pro Leu Cys Ile Thr Met Arg
            100                 105                 110
Cys Asn Lys Ser Glu Thr Asp Arg Trp Gly Leu Thr Lys Ser Ile Thr
        115                 120                 125
Thr Thr Ala Ser Thr Thr Ser Thr Thr Ala Ser Ala Lys Val Asp Met
    130                 135                 140
Val Asn Glu Thr Ser Ser Cys Ile Ala Gln Asp Asn Cys Thr Gly Leu
145                 150                 155                 160
Glu Gln Glu Gln Met Ile Ser Cys Lys Phe Asn Met Thr Gly Leu Lys
                165                 170                 175
Arg Asp Lys Lys Lys Glu Tyr Asn Glu Thr Trp Tyr Ser Ala Asp Leu
            180                 185                 190
Val Cys Glu Gln Gly Asn Asn Thr Gly Asn Glu Ser Arg Cys Tyr Met
        195                 200                 205
```

```
Asn His Cys Asn Thr Ser Val Ile Gln Glu Ser Cys Asp Lys His Tyr
    210                 215                 220
Trp Asp Ala Ile Arg Phe Arg Tyr Cys Ala Pro Pro Gly Tyr Ala Leu
225                 230                 235                 240
Leu Arg Cys Asn Asp Thr Asn Tyr Ser Gly Phe Met Pro Lys Cys Ser
                245                 250                 255
Lys Val Val Val Ser Ser Cys Thr Arg Met Met Glu Thr Gln Thr Ser
            260                 265                 270
Thr Trp Phe Gly Phe Asn Gly Thr Arg Ala Glu Asn Arg Thr Tyr Ile
        275                 280                 285
Tyr Trp His Gly Arg Asp Asn Arg Thr Ile Ile Ser Leu Asn Lys Tyr
    290                 295                 300
Tyr Asn Leu Thr Met Lys Cys Arg Arg Pro Gly Asn Lys Thr Val Leu
305                 310                 315                 320
Pro Val Thr Ile Met Ser Gly Leu Val Phe His Ser Gln Pro Ile Asn
                325                 330                 335
Asp Arg Pro Lys Gln Ala Trp Cys Trp Phe Gly Gly Lys Trp Lys Asp
            340                 345                 350
Ala Ile Lys Glu Val Lys Gln Thr Ile Val Lys His Pro Arg Tyr Thr
        355                 360                 365
Gly Thr Asn Asn Thr Asp Lys Ile Asn Leu Thr Ala Pro Gly Gly Gly
    370                 375                 380
Asp Pro Glu Val Thr Phe Met Trp Thr Asn Cys Arg Gly Glu Phe Leu
385                 390                 395                 400
Tyr Cys Lys Met Asn Trp Phe Leu Asn Trp Val Glu Asp Arg Asn Thr
                405                 410                 415
Ala Asn Gln Lys Pro Lys Glu Gln His Lys Arg Asn Tyr Val Pro Cys
            420                 425                 430
His Ile Arg Gln Ile Ile Asn Thr Trp His Lys Val Gly Lys Asn Val
        435                 440                 445
Tyr Leu Pro Pro Arg Glu Gly Asp Leu Thr Cys Asn Ser Thr Val Thr
    450                 455                 460
Ser Leu Ile Ala Asn Ile Asp Trp Ile Asp Gly Asn Gln Thr Asn Ile
465                 470                 475                 480
Thr Met Ser Ala Glu Val Ala Glu Leu Tyr Arg Leu Glu Leu Gly Asp
                485                 490                 495
Tyr Lys Leu Val Glu Ile Thr Pro Ile Gly Leu Ala Pro Thr Asp Val
            500                 505                 510
Lys Ar. Tyr Thr Thr Gly Gly Thr Ser Arg Asn Lys Arg Gly Val Phe
        515                 520                 525
Val Leu Gly Phe Leu Gly Phe Leu Ala Thr Ala Gly Ser Ala Met Gly
    530                 535                 540
Ala Ala Ser Leu Thr Leu Thr Ala Gln Ser Arg Thr Leu Leu Ala Gly
545                 550                 555                 560
Ile Val Gln Gln Gln Gln Gln Leu Leu Asp Val Val Lys Arg Gln Gln
                565                 570                 575
Glu Leu Leu Arg Leu Thr Val Trp Gly Thr Lys Asn Leu Gln Thr Arg
            580                 585                 590
```

```
Val Thr Ala Ile Glu Lys Tyr Leu Lys Asp Gln Ala Gln Leu Asn Ala
        595                 600                 605
Trp Gly Cys Ala Phe Arg Gln Val Cys His Thr Thr Val Pro Trp Pro
    610                 615                 620
Asn Ala Ser Leu Thr Pro Lys Trp Asn Asn Glu Thr Trp Gln Glu Trp
625                 630                 635                 640
Glu Arg Lys Val Asp Phe Leu Glu Glu Asn Ile Thr Ala Leu Leu Glu
                645                 650                 655
Glu Ala Gln Ile Gln Gln Glu Lys Asn Met Tyr Glu Leu Gln Lys Leu
            660                 665                 670
Asn Ser Trp Asp Val Phe Gly Asn Trp Phe Asp Leu Ala Ser Trp Ile
        675                 680                 685
Lys Tyr Ile Gln Tyr Gly Val Tyr Ile Val Val Gly Val Ile Leu Leu
    690                 695                 700
Arg Ile Val Ile Tyr Ile Val Gln Met Leu Ala Lys Leu Arg Gln Gly
705                 710                 715                 720
Tyr Arg Pro Val Phe Ser Ser Pro Pro Ser Tyr
                725                 730
```

CLAIMS

1. An immunotherapeutic method of treating a host having or at risk of having a lentivirus infection, comprising administering to the host a therapeutically effective amount of an attenuated virus comprising a retrovirus nucleotide sequence encoding a macrophage-tropic polypeptide which stimulates an immune response.

2. The method of claim 1, wherein the retrovirus is a lentivirus.

3. The method of claim 2, wherein the lentivirus is simian immunodeficiency virus (SIV).

4. The method of claim 2, wherein the lentivirus is human immunodeficiency virus (HIV).

5. The method of claim 4, wherein the HIV is HIV type 1 (HIV-1).

6. The method of claim 1, wherein the attenuated virus is a DNA virus.

7. The method of claim 6, wherein the virus is selected from the group consisting of adenovirus, herpes virus and vaccinia virus.

8. The method of claim 1, wherein the attenuated virus is an RNA virus.

9. The method of claim 8, wherein the virus is a retrovirus.

10. The method of claim 1, wherein the attenuated virus is a non-pathogenic virus.

11. The method of claim 1, wherein the retrovirus nucleotide sequence encoding a macrophage-tropic polypeptide encodes a virus envelope (*env*) polypeptide.

12. The method of claim 11, wherein the virus envelope polypeptide is HIV envelope.

13. The method of claim 11, wherein the virus envelope polypeptide is SIV envelope.

14. The method of claim 11, wherein the retrovirus nucleotide sequence encodes gp120.

15. The method of claim 11, wherein the retrovirus nucleotide sequence encodes gp120 and about 189 amino acids from the amino terminus of gp41.

16. The method of claim 1, wherein the attenuated virus is a recombinant virus.

17. The method of claim 1, wherein the attenuated virus is a whole inactivated virus.

18. The method of claim 1, wherein the host is a non-human primate.

19. The method of claim 1, wherein the host is a human.

20. An immunotherapeutic method of treating a host having or at risk of having a lentivirus infection, comprising administering to the host a therapeutically effective amount of vehicle comprising a retrovirus nucleotide sequence encoding a macrophage-tropic polypeptide which stimulates an immune response.

21. The method of claim 20, wherein the retrovirus is a lentivirus.

22. The method of claim 21, wherein the lentivirus is simian immunodeficiency virus (SIV).

23. The method of claim 21, wherein the lentivirus is human immunodeficiency virus (HIV).

24. The method of claim 23, wherein the HIV is HIV type 1 (HIV-1).

25. The method of claim 20, wherein the retrovirus nucleotide sequence encoding a macrophage-tropic polypeptide encodes a virus envelope (*env*) polypeptide.

26. The method of claim 25, wherein the virus envelope polypeptide is HIV envelope.

27. The method of claim 25, wherein the virus envelope polypeptide is SIV envelope.

28. The method of claim 25, wherein the retrovirus nucleotide sequence encodes gp120.

29. The method of claim 25, wherein the retrovirus nucleotide sequence encodes gp120 and about 189 amino acids from the amino terminus of gp41.

30. The method of claim 20, wherein the vehicle is a colloidal dispersion system.

31. The method of claim 30, wherein the colloidal dispersion system is a liposome.

32. The method of claim 20, wherein the vehicle is a virus.

33. The method of claim 32, wherein the virus is a DNA virus.

34. The method of claim 33, wherein the virus is selected from the group consisting of adenovirus, herpesvirus, and vaccinia virus.

35. The method of claim 32, wherein the virus is an RNA virus.

36. The method of claim 35, wherein the virus is a retrovirus.

37. A pharmaceutical composition comprising an attenuated virus comprising a retrovirus nucleotide sequence encoding a macrophage-tropic polypeptide which stimulates an immune response, in a pharmaceutically acceptable carrier.

38. The composition of claim 37, wherein the retrovirus nucleotide sequence encoding a macrophage-tropic polypeptide encodes a virus envelope (*env*) polypeptide.

39. The composition of claim 38, wherein the virus envelope polypeptide is HIV envelope.

40. The composition of claim 38, wherein the composition comprises a nucleotide sequence encoding an HIV envelope protein in operable linkage in a lentivirus genome.

41. The composition of claim 40, wherein the lentivirus is SIV.

42. The composition of claim 41, wherein the SIV is macrophage-tropic.

43. The composition of claim 41, wherein the SIV is lymphocyte-tropic.

44. The composition of claim 40, wherein the lentivirus is HIV.

45. The composition of claim 44, wherein the HIV is macrophage-tropic.

46. The composition of claim 44, wherein the HIV is lymphocyte-tropic.

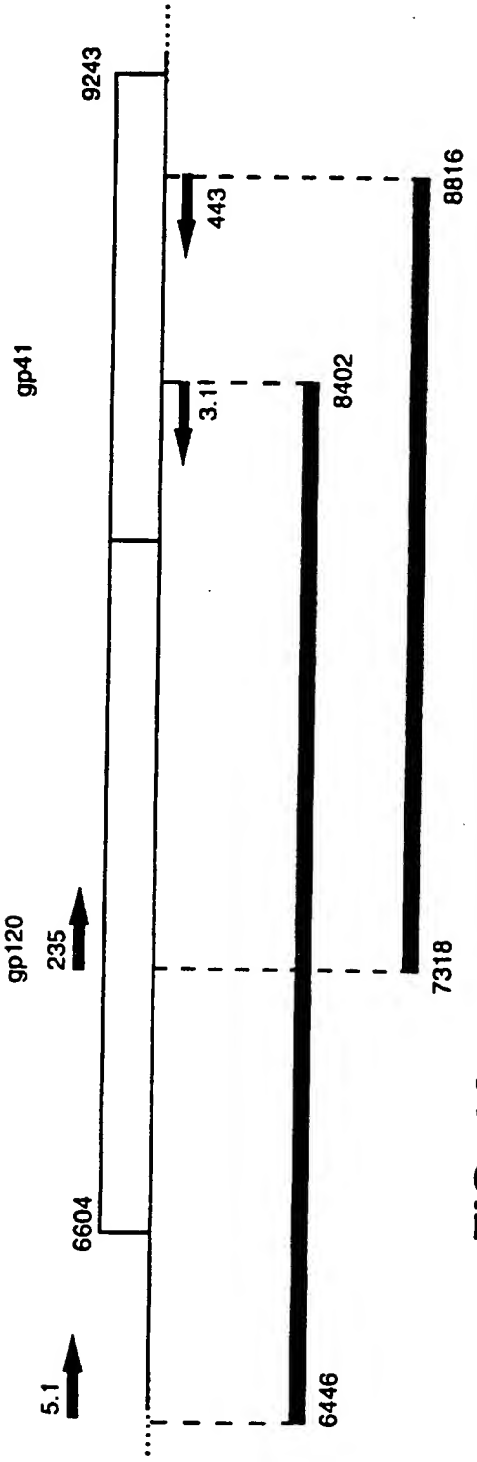

FIG. 1A

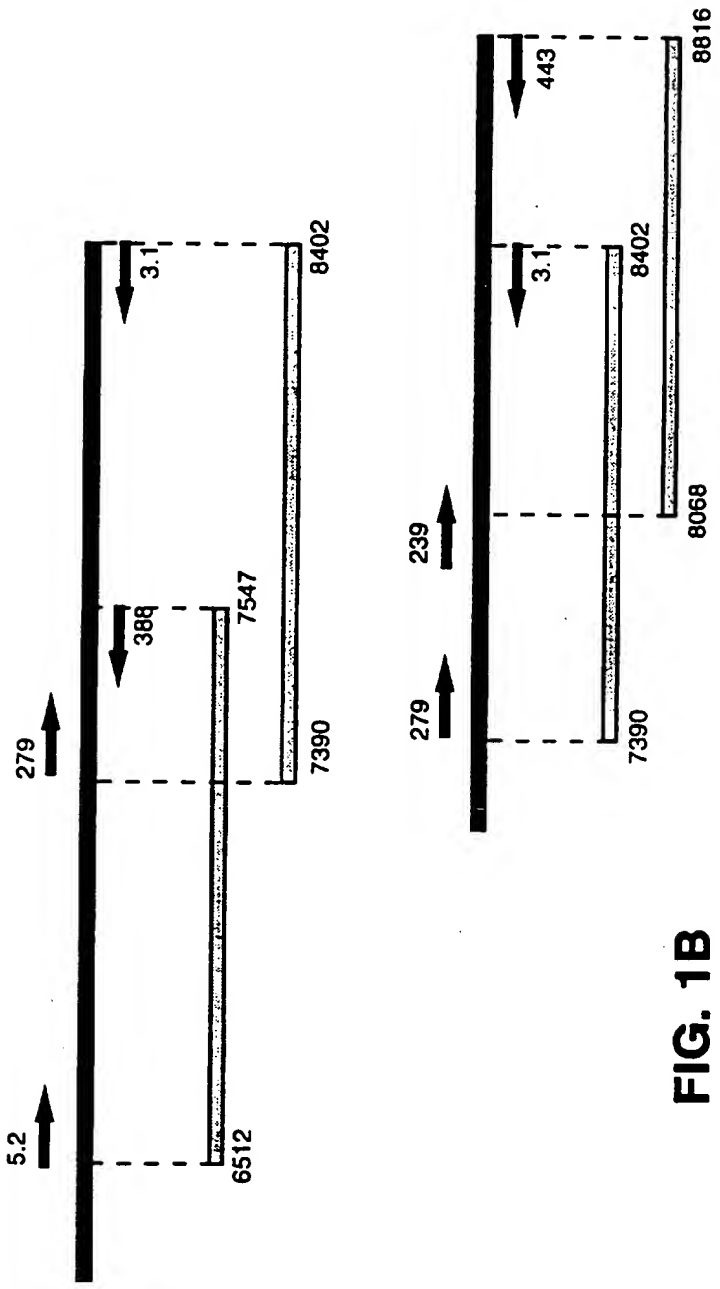
5.2
279
6512
388
7547
3.1
8402
7390
279
239
443
8816
3.1
8402
8068
7390

FIG. 1B

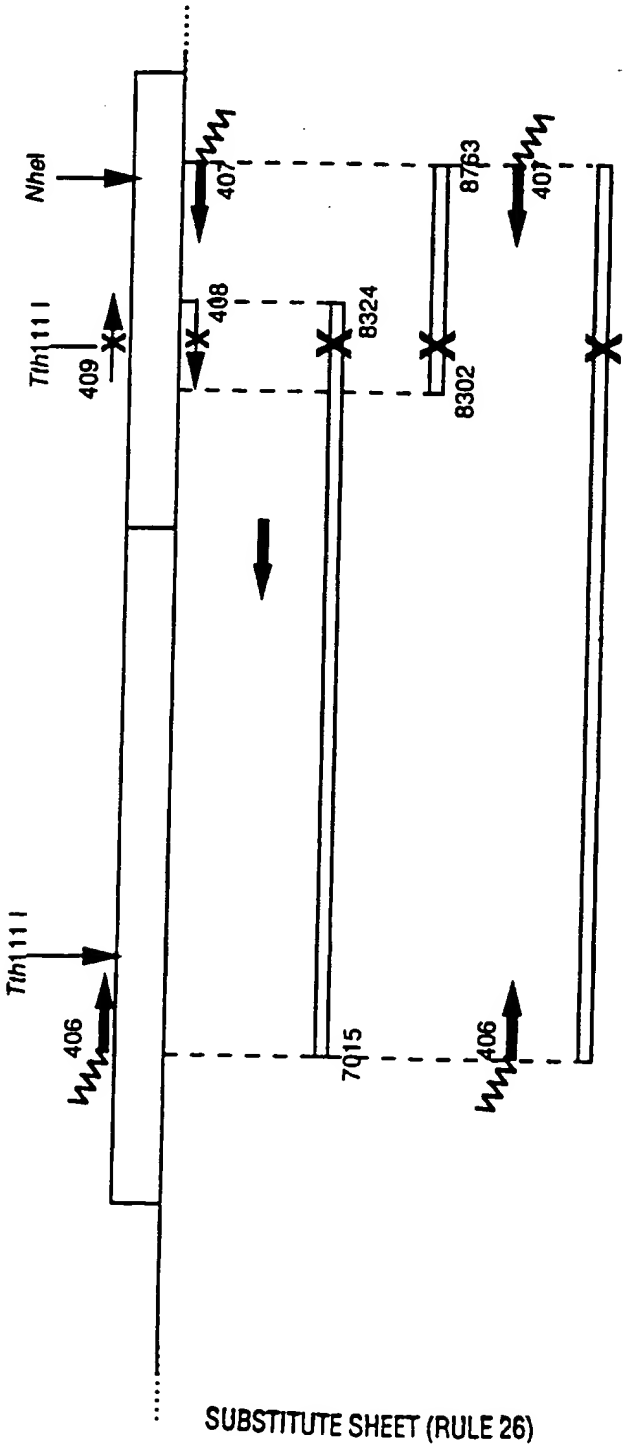
NheI
TthIIII
TthIIII
407
408
409
406
406
407
407
8763
8324
8302
7915

FIG. 1C

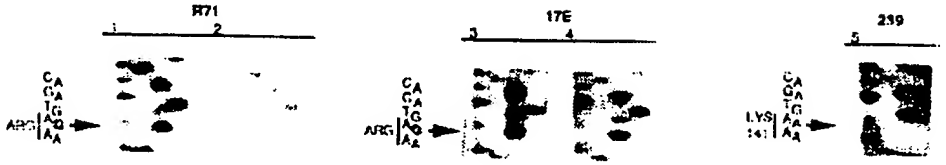

FIG. 2A

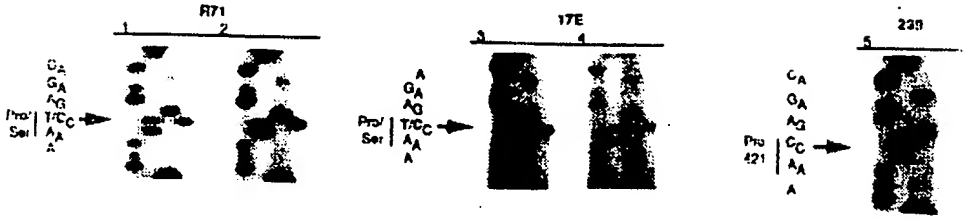

FIG. 2B

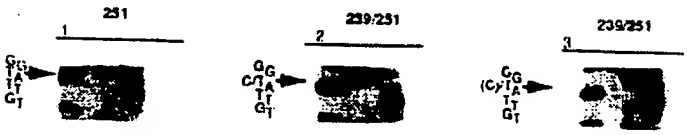

FIG. 2C

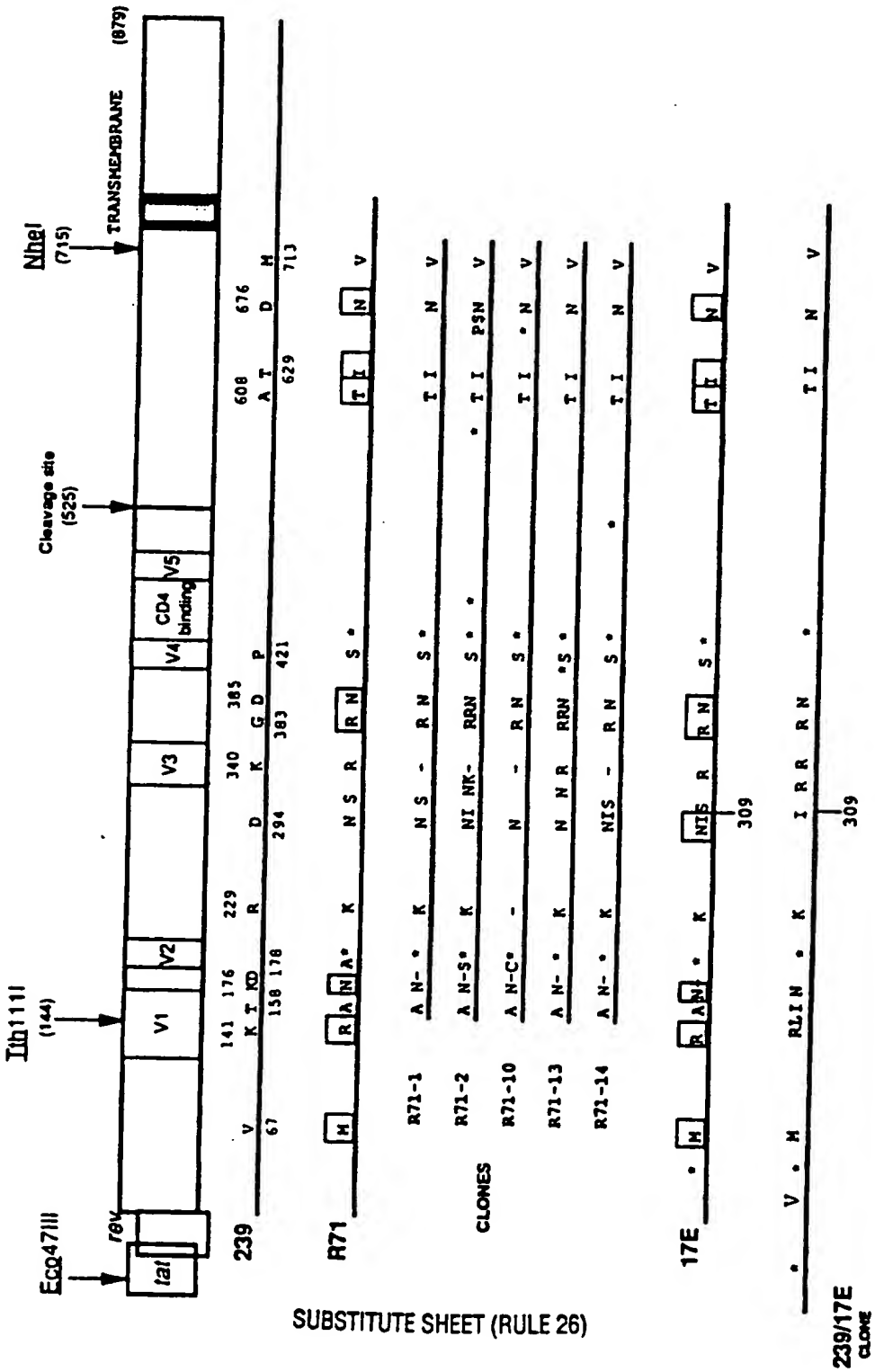
Eco47III
Ith111I
(144)
NheI
(715)
Cleavage site
(525)
TRANSMEMBRANE
(879)
rev
tat
V1
V2
V3
V4
V5
CD4 binding
239
R71
CLONES
R71-1
R71-2
R71-10
R71-13
R71-14
17E
239/17E
CLONE

FIG. 3

```
                10                                                  50
239  MGCLGNQLLI AILLLSVYGI YCTLYVTVFY GVPAWRNATI PLFCATKNRD TWGTTQCLPD
R71-1---------- ---------- ---------- ---------- ---------- ----------
R71CL---------- ---------- ---------- ---------- ---------- ----------
17E  ---------- ---------- ---------- ---------- -*-------- ----------
17ECL---------- ---------- ---------- ---V------ -*-------- ----------
316  ---------- ---------- ---------- ---------- ---------- ----------
          120                  V1              150
239  MRCNKSETDR WGLTKSITTT ASTTSTTASA KVDMVNETSS CIAQDNCTGL EQEQMISCKF
R71-1---------- ---------- ---------- R--------- -------A-- ----------
R71CL---------- ---------- ---------- ---------- -------A-- ----------
17E  ---------- ---------- ---------- R--------- -------A-- ----------
17ECL---------- ---------- ---------- R--------- -------A-- ----------
316  ---------- ---------- ---------- ---------- ---------- ----------
                     250
239  DKHYWDAIRF RYCAPPGYAL LRCNDTNYSG FMPKCSKVVV SSCTRMMETQ TSTWFGFNGT
R71-1--------K- ---------- ---------- ---------- ---------- ----------
R71CL--------K- ---------- ---------- ---------- ---------- ----------
17E  --------K- ---------- ---------- ---------- ---------- ----------
17ECL--------K- ---------- ---------- ---------- ---------- ----------
316  ---------- ---------- ---------- ---------- ---------- ----------
       V3  340      350
239  HSQPINDRPK QAWCWFGGKW KDAIKEVKQT IVKHPRYTGT NNTDKINLTA PGGGDPEVTF
R71-1---------R ---------- ---------- ---------- ---------- --R-N-----
R71CL---------R ---------- ---------- ---------- ---------- --R-N-----
17E  ---------R ---------- ---------- ---------- ---------- --R-N-----
17ECL---R-----R ---------- ---------- ---------- ---------- --R-------
316  ---------- ---------- ---------- ---------- ---------- --R-------
          450                          V5                        500
239  WHKVGKNVYL PPREGDLTCN STVTSLIANI DWIDGNQTNI TMSAEVAELY RLELGDYKLV
R71-1---------- ---------- ---------- ---------- ---------- ----------
R71CL---------- ---------- ---------- ---------- ---------- ----------
17E  ---------- ---------- ---------- ---------- ---------- ----------
17ECL---------- ---------- ---------- ---------- ---------- ----------
316  ---------- ---------- ---------- ---------- ---------- ----------
          560                                            600
239  TAQSRTLLAG IVQQQQQLLD VVKRQQELLR LTVWGTKNLQ TRVTAIEKYL KDQAQLNAWG
R71-1---------- ---------- ---------- ---------- ---------- -------T--
R71CL---------- ---------- ---------- ---------- ---------- -------T--
17E  ---------- ---------- ---------- ---------- ---------- -------T--
17ECL---------- ---------- ---------- ---------- ---------- -------T--
316  ---------- ---------- --T------- ---------- ---------- ----------
          670                              700
239  QQEKNMYELQ KLNSWDVFGN WFDLASWIKY IQYGVYIVVG VILLRIVIYI VQMLAKLRQG
R71-1---------- -----N---- ---------- ---------- ---------- --V-------
R71CL---------- -----N---- ---------- ---------- ---------- --V-------
17E  ---------- -----N---- ---------- ---------- ---------- --V-------
17ECL---------- -----N---- ---------- ---------- ---------- --V-------
316  ---------- ---------- ---------- ---------- ---------- ----------
```

FIG. 4A

```
                                                          100
239   NGDYSEVALN VTESFDAWNN TVTEQAIEDV WQLFETSIKP CVKLSPLCIT
R71-1 ------M--- ---------- ---------- ---------- ----------
R71CL ------M--- ---------- ---------- ---------- ----------
17E   ------M--- ---------- ---------- ---------- ----------
17ECL ------M--- ---------- ---------- ---------- ----------
316   ------M--- ---------- ---------- ---------- ----------
                      V2          200
239   NMTGLKRDKK KEYNETWYSA DLVCEQGNNT GNESRCYMNH CNTSVIQESC
R71-1 -----N-A-- -----*---- ---------- ---------- ----------
R71CL -----N---- -----*---- ---------- ---------- ----------
17E   -----N---- -----*---- ---------- ---------- ----------
17ECL -----N---- -----*---- ---------- ---------- ----------
316   -----E---- ---------- ---------- ---------- ----------
          300                                          .
239   RAENRTYIYW HGRDNRTIIS LNKYYNLTMK CRRPGNKTVL PVTIMSGLVF
R71-1 ---------- ---N------ ---------- ---------S ----------
R71CL ---------- ---N------ ---------- ---------S ----------
17E   ---------- ---N------ --------I- ---------S ----------
17ECL ---------- ---------- --------I- ---------- ----------
316   ---------- ---------- ---------- ---------- ----------
            400          V4
239   MWTNCRGEFL YCKMNWFLNW VEDRNTANQK PKEQHKRNYV PCHIRQIINT
R71-1 ---------- ---------- ---------- S--*------ ----------
R71CL ---------- ---------- ---------- S--*------ ----------
17E   ---------- ---------- ---------- S--*------ ----------
17ECL ---------- ---------- ---------- ---*------ ----------
316   ---------- ---------- ---------- ---------- ----------
                                                        550
239   EITPIGLAPT DVKRYTTGGT SRNKRGVFVL GFLGFLATAG SAMGAASLTL
R71-1 ---------- ---------- ---------- ---------- ----------
R71CL ---------- ---------- ---------- ---------- ----------
17E   ---------- ---------- ---------- ---------- ----------
17ECL ---------- ---------- ---------- ---------- -------*--
316   ---------- ---------- ---------- ---------- ----------
                                              650
239   CAFRQVCHTT VPWPNASLTP KWNNETWQEW ERKVDFLEEN ITALLEEAQI
R71-1 ---------- --------I- ---------- ---------- ----------
R71CL ---------- --------I- ---------- ---------- ----------
17E   ---------- --------I- ---------- ---------- ----------
17ECL ---------- --------I- ---------- ---------- ----------
316   ---------- ---------- ---------- ---------- ----------
          730
239   YRPVFSSPPS Y....
R71-1 ---------- -
R71CL ---------- -
17E   ---------- -
17ECL ---------- -
316   ---------- -
```

FIG. 4B

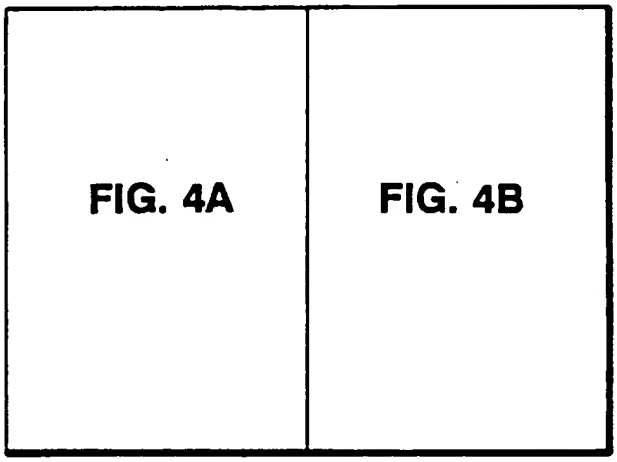

FIG. 4C

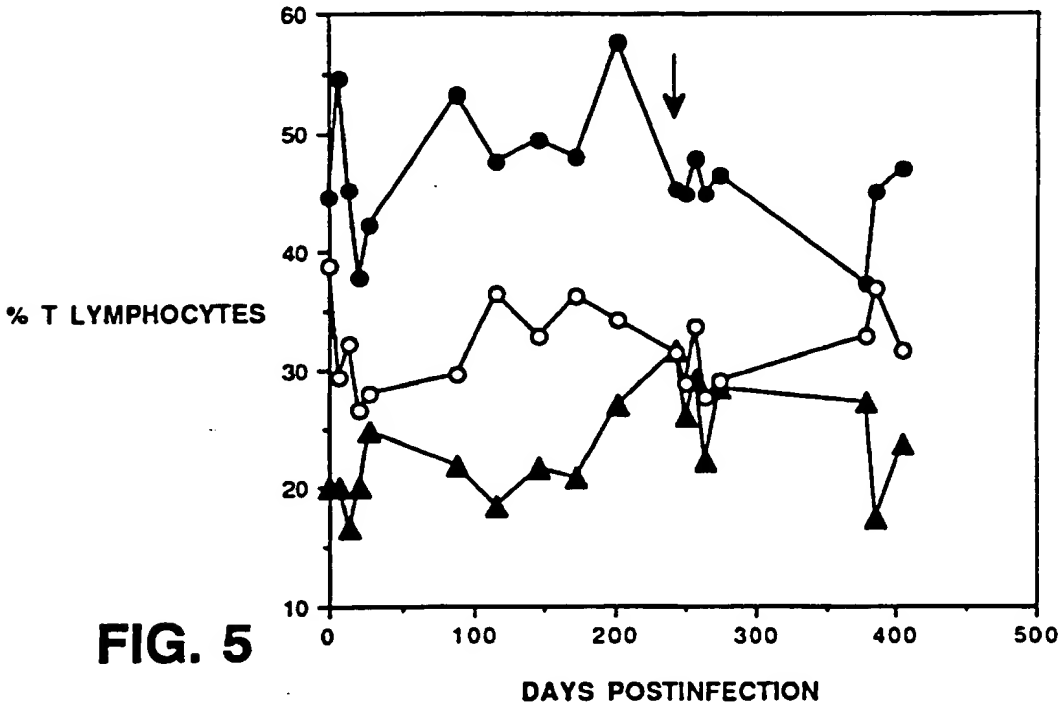
% T LYMPHOCYTES
60
50
40
30
20
10
0
100
200
300
400
500
DAYS POSTINFECTION

FIG. 5

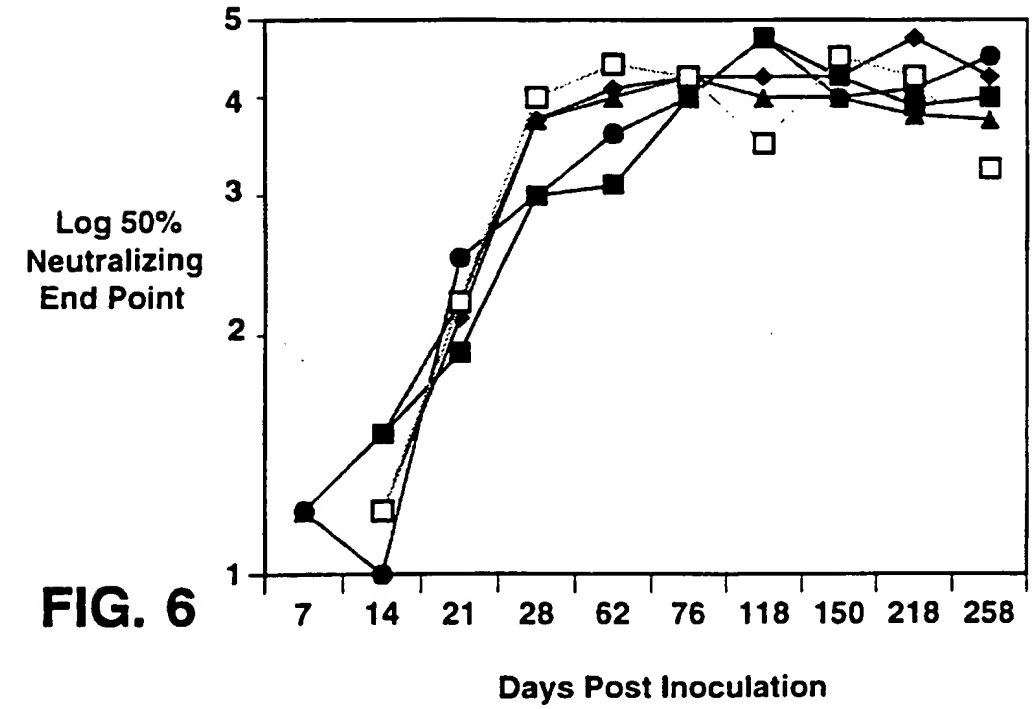
Log 50%
Neutralizing
End Point
5
4
3
2
1
7
14
21
28
62
76
118
150
218
258
Days Post Inoculation

FIG. 6

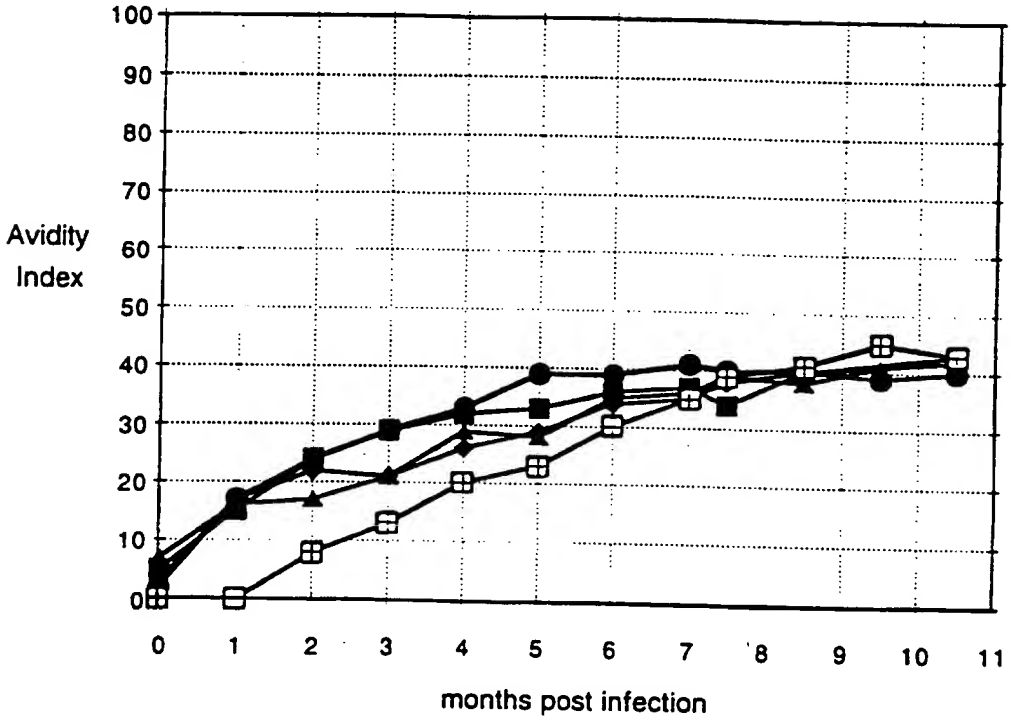
Avidity Index
100
90
80
70
60
50
40
30
20
10
0
0 1 2 3 4 5 6 7 8 9 10 11
months post infection

FIG. 7

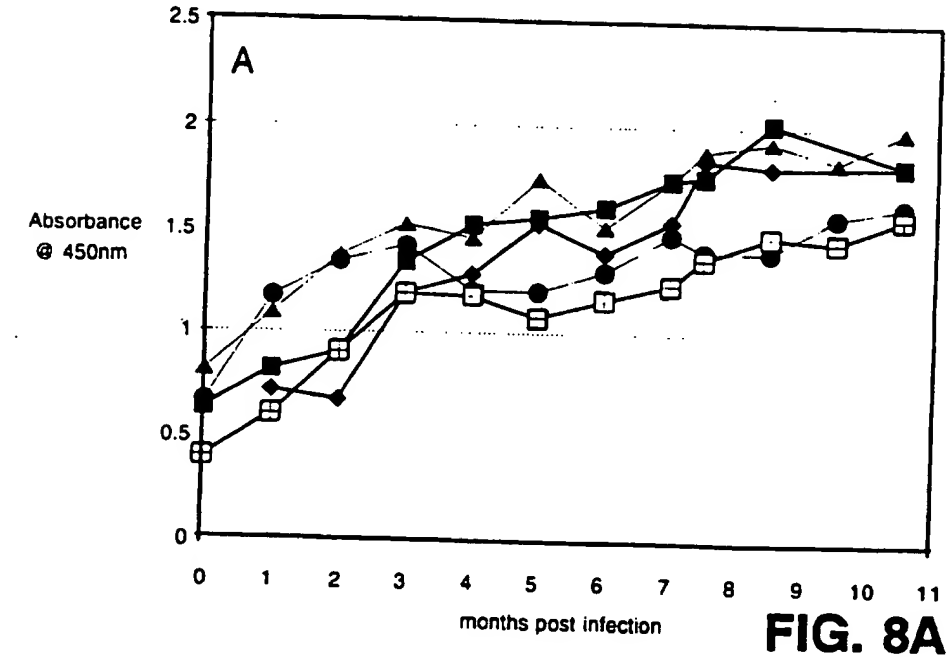
A
Absorbance
@ 450nm
2.5
2
1.5
1
0.5
0
0 1 2 3 4 5 6 7 8 9 10 11
months post infection

FIG. 8A

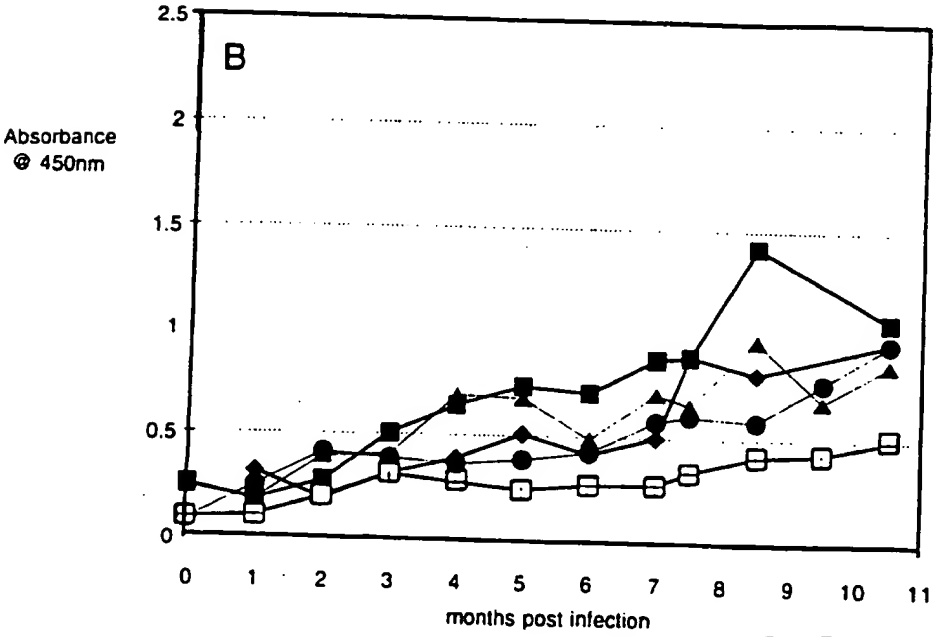
B
Absorbance
@ 450nm
2.5
2
1.5
1
0.5
0
0 1 2 3 4 5 6 7 8 9 10 11
months post infection

FIG. 8B

Antigen-Specific CTL and Antibody Responses in Monkeys Immunized with nef-Deleted Monocytes- and Lymphocyte-Tropic SIV Clones

SIV	Animal No.	Lytic Unit 15%/10^7 Cells						ELISA gp140 Titers at 8 Months
		6 Months			8 Months			
		env	gag	pol	env	gag	pol	
17E Δ nef	M801	ND	ND	ND	-	-	-	400
	M810	-	144	9	-	-	-	50
	M901	24	16	13	20	13	-	800
	N153	-	-	-	51	90	29	100
	N245	ND	ND	ND	-	-	-	1600
	N338	ND	ND	ND	-	-	-	100
239 Δ nef	L790	ND	ND	ND	-	-	-	3200
	N182	-	27	-	-	-	-	3200
	N183	-	-	-	-	-	-	6400
	N184	-	-	-	-	-	-	6400
	N185	-	-	-	-	-	-	6400
	N186	ND	ND	ND	-	-	-	3200

$$\frac{\text{No. of purified CD8+}}{\text{Target cell\# x (estimated E:T ratio)}} \quad \text{Original culture cell\#}$$

FIG. 9